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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION. PRECIPITATION-DISSOLUTION REACTION IN MIXTURES OF ACTIVE VIRUS AND INHIBITOR.

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In previous papers (Svedmyr, 1947*a*, 1947*b*, 1948*a*, 1948*b* ; Gard, von Magnus and Svedmyr, 1947) the haemagglutination inhibitor was described which is present in normal allantoic fluid obtained from chick embryos. The observations indicated that the inhibitive capacity of normal allantoic fluid on influenza virus haemagglutination is connected with particles having a sedimentation constant of about 200 S, and that the inhibitor acts by combining with the virus particles. In high concentrations the reaction manifested itself as a macroscopic flocculation.

Active virus, even in small quantities, was shown to have the capacity, when incubated at 35° C., with normal allantoic fluid, to destroy gradually the inhibitory activity. After sufficient destruction, or modification, of the inhibitor particles, haemagglutinins appeared in the previously non-haemagglutinating mixture.

By suitable treatment it was possible to destroy successively the capacity of the virus particle (*a*) to infect, (*b*) to modify the inhibitor, and (*c*) to combine with the inhibitor and to haemagglutinate.

Certain of these previously reported results have been confirmed by the work of Hardy and Horsfall (1948).

The purpose of this investigation was to study the virus-inhibitor precipitation in some detail.

MATERIALS AND METHODS.

Virus Preparations.

The PR8 strain of influenza virus was used throughout these experiments. The virus was propagated in the allantoic cavity of 11-day-old chick embryos. Two types of virus preparations were employed : the classical " standard virus "

(SV) with a sedimentation constant of about 700S, and the "modified virus" (MV) obtained by three allantoic passages of undiluted allantoic fluid, its main component having a sedimentation constant around 500 S (von Magnus, 1947*a*, 1947*b*; Gard and von Magnus, 1947; Gard, von Magnus and Svedmyr, 1947; Svedmyr, 1948*b*).

Purification.

Virus concentrates were prepared by differential centrifugation in two cycles of allantoic fluid clarified by suction-filtration through tightly-packed cotton-wool. The first cycle included sedimentation and washing in a refrigerated Sharples centrifuge operating at 48,000–50,000 r.p.m. with a rate of flow of about 1·4 litres per hour, followed by clarification in an angle centrifuge at 5000 r.p.m. for 5 minutes. The second cycle consisted of sedimentation in an air-driven centrifuge at 27,000 r.p.m. for 20 minutes, and a further low speed centrifugation at 5000 r.p.m. for 5 minutes. The final virus concentrate was made up with phosphate buffer to 1/1000 of the starting volume of allantoic fluid.

In the MV preparation used this general procedure was slightly modified. The clarification of the Sharples concentrate was performed by centrifugation in an "Ecco," bucket-type centrifuge at 4500 r.p.m. for 10 minutes, the second high speed centrifugation was run for 90 minutes instead of 20, and the final clarification was performed by spinning up-and-down to 10,000 in the air-driven centrifuge. The concentration by volume was about 900 times that of the starting material. This preparation was kindly supplied by Dr. P. von Magnus.

The nitrogen contents of the preparations used in the present experiments were as follows: SV1—4·3 mg. per ml.; SV2—4·0 mg. per ml.; MV—1·48 mg. per ml. The biological properties of SV1 and MV are listed in Tables I and II.

Table I shows that SV1 contained 50 per cent or more of the activity of the starting material, as measured both by infectivity, haemagglutination titre and inhibitor-destroying capacity. The term "active virus" means virus that has retained its capacity to destroy the inhibitor, the infectivity being irrelevant in this connection.

Inhibitor Preparations.

Two preparations of inhibitor were used in these experiments. The starting material was one batch of normal allantoic fluid from embryos 13 days old and one pool from embryos 14 days old. The purification methods were essentially the same for the two batches. After clarification the fluid was diluted to about double the volume with buffered saline in order to minimize precipitation of uric acid during the next step. The fluid was then concentrated to about 1/25 of the original volume by means of ultrafiltration. The concentrate was subjected to two cycles of differential centrifugation. First, 27,000 r.p.m. for 90 minutes, followed by clarification in an angle centrifuge at 5000 r.p.m. for 10 minutes for Batch 1 and spinning up-and-down to 10,000 r.p.m. in the air-driven centrifuge for Batch 2; then a further 60 minutes at 27,000 r.p.m., and a final low speed centrifugation at 5000 r.p.m. for 15 minutes. The final concentrate was made up with phosphate buffer to 1/1000 of the original volume. Both preparations contained 0·51 mg. nitrogen per ml. Titres of the inhibitory activity of the different fractions are listed in Table III.

TABLE I.—*Properties of the Various Fractions from the Differential Centrifugation of SV Preparation* 1.

Virus material.			Allantoic fluid.				Supernatant from Sharples centrifugation.		Sharples sediment.		Supernatant 5000 I.
50 per cent chick embryo infectivity endpoint (3 eggs per group)			7·8				..		..		..
Haemagglutination titre			4·09				2·93		5·36		5·44
Residual inhibitory activity of normal allantoic fluid incubated with the virus*	Concentration of virus.		1/100	1/300	1/900	1/2700	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-3}
	Hours of incubation	0	0·83	1·06	0·98	1·13	0·91	0·98	0·45	1·06	0·38
		3	0·45	0·91	0·98	1·13	0·53	0·98	+	0·91	+
		20	+	0·38	0·68	0·98	+	0·75	+	0·30	+

+ = Positive haemagglutination of the tested fluid.

* Normal fluid was incubated at 35° C. with different amounts of the virus. Samples were removed after various periods of time and tested for residual inhibitory activity after heating in a 65° C. water bath for 15 minutes.

Virus material.			Sediment 5000 I.	Supernatant 27,000.	Sediment 27,000.	Supernatant 5000 II = conc.			Sediment 5000 II.	
50 per cent chick embryo infectivity endpoint (3 eggs per group)			..	..	..	11·5			..	..
Haemagglutination titre			4·51	2·78	6·71	6·78			4·99	..
Residual inhibitory activity of normal allantoic fluid incubated with the virus.*	Concentration of virus.		10^{-2}	..	..	10^{-4}	10^{-5}	10^{-6}	..	Control without virus.
	Hours of incubation	0	0·08	..	..	+	0·98	1·06	..	0·98
		3	+	..	..	+	0·83	1·13	..	..
		20	+	..	..	+	+	0·98	..	0·68

TABLE II.—*Properties of the MV Preparation.*

Virus material.			"3. passage" allantoic fluid.	Supernatant. 10,000 = final concentrate.			Control: SV Preparation 1.			
50 per cent chick embryo infectivity endpoint			4·9	6·9			..			..
Haemagglutination titre			3·33	5·97			..			..
Residual inhibitory activity of normal allantoic fluid incubated with the virus*	Concentration of virus.		..	1/10,000	1/40,000	1/160,000	1/50,000	1/200,000	1/800,000	Control without virus.
	Hours of incubation	0	..	0·83	0·98	0·98	0·83	1·06	1·06	1·13
		3	..	0·38	0·38	0·68	−0·07	0·83	1·06	..
		20	..	0·08	0·23	0·38	+	−0·07	0·38	0·98

+ = Positive haemagglutination of the tested fluid.

* See note, Table I.

TABLE III.—*Titre Values of the Inhibitory Activity of the Various Fractions from the Purification of Inhibitor.*

Inhibitor fraction.	Inhibitory activity.*	
	Preparation 1.	Preparation 2.
Original normal allantoic fluid	1·43	1·13
Same diluted by phosphate buffer	1·13	1·13
Ultrafiltrate	—0·07	—0·07
Ultraconcentrate	0·83	..
Supernatant 27,000 I	0·23	0·38
Sediment 27,000 I	0·53	..
Supernatant 5000 I	0·53	..
Sediment 5000 I or 10,000 respectively	—0·07	0·23
Supernatant 27,000 II	—0·07	0·08
Sediment 27,000 II	0·75	0·53
Supernatant 5000 II	0·83	0·83
Sediment 5000 II	0	0·08

* The inhibitory activity was determined in the usual way after dilution of the test material corresponding to the volume of the starting material.

Most of the preparations and reaction mixtures from the present experiments were analysed in the ultracentrifuge. The results will be reported in detail elsewhere. It should only be mentioned that the SV and MV appeared pure in the centrifuge. The inhibitor preparations in addition to the "200 S" component contained a homogeneous slow-sedimenting component ("30 S").

Preservatives.

The use of merthiolate, employed in earlier experiments (Svedmyr, 1948*a*, 1948*b*) as preservative, has been found to cause a certain loss of virus activity during purification. Instead, 100 units of crystalline penicillin and 100 γ of streptomycin per ml. was added to all concentrates.

Buffer solutions.

0·1 M phosphate buffer with a pH of 7·0 was used. Inhibitor Concentrate 2, however, was taken up in a mixture of 80 per cent saline and 20 per cent of this buffer.

Saline.

0·9 per cent NaCl in distilled water was used.

Titration Methods.

Haemagglutination tests.

The methods were described in detail and their accuracy evaluated in a previous paper (Svedmyr, 1948*a*). All titre values are recorded as the logarithm of the final virus dilution.

Inhibitory activity.—Red cells from a minority of fowls gave lower titres when used in the titrations for inhibitory activity, which agrees with the observations

of Anderson (1948). In these experiments only cells giving high inhibitor titres were used.

The inhibitory activity of a test material was usually determined as previously reported (Svedmyr, 1948*a*), i.e. serial dilutions of an active standard virus allantoic fluid were made up with the inhibitor material as diluent, and the difference between the log titre value obtained and the control titre of the virus when diluted in saline was recorded.

In some experiments, however, where very many titrations had to be performed on the same day, a somewhat modified technique was employed. To 0·25 ml. amounts of the inhibitor material 0·25 ml. of each virus dilution was added and, finally, 0·5 ml. of red cell suspension. Stock dilutions of virus could thus be prepared at one time for a series of titrations. When corrected for different virus and inhibitor amounts no significant difference in titres was observed with the two methods, i.e. within the range of activity employed in the present experiments.

Inhibitor-destroying capacity.—The inhibitor-destroying capacity of a virus suspension was tested as reported previously (Svedmyr, 1948*b*); normal allantoic fluid was incubated at 35° C. with small amounts of the virus. Samples were removed after different periods of time and tested for residual inhibitory activity after being heated in the water bath at 65° C. for 15 minutes in order to interrupt the process.

Chick embryo titrations.

Chick embryo titrations were performed as previously described (Svedmyr, 1948*b*).

EXPERIMENTAL.

It was previously reported (Svedmyr, 1947*b*, 1948*b*) that the precipitate appearing in a mixture of purified concentrates of influenza virus and inhibitor seemed to be rather stable even after incubation at 35° C. for one hour, and it was assumed that the virus suspension had lost most of its inhibitor-destroying capacity, possibly since it had been stored for a long time with 1/10,000 merthiolate as preservative. As a matter of fact, quite different results were obtained with the active virus preparations described here.

In the present experiments the volume of the reaction mixtures varied from 2·5 ml. in tests intended for analysis in the ultracentrifuge and for haemagglutination to 0·05 ml., which was found sufficient for observation of the flocculation with the aid of a magnifying-glass. When mixtures had to be prepared at low temperatures reagents as well as glassware were pre-chilled.

Precipitation phase.

Under the conditions mentioned the SV preparations formed heavy precipitates when mixed with inhibitor in the proportions 1+4, leaving an almost clear supernatant after storage in the cold. The MV preparation, being less concentrated, gave a rather slight precipitate when mixed in the same proportions, presumably with free inhibitor in the supernatant. When mixed with inhibitor in equal amounts, however, the MV concentrate also gave a heavy flocculation and a comparatively clear supernatant.

Dissolution phase.

When the tubes containing these heavy precipitates were placed in an incubator at 35° C. or only warmed in the hand, the suspension cleared up almost completely in about 2 minutes, and after 5–10 minutes no floccules could be seen with a magnifying-glass. In the first 5 minutes in the incubator the temperature in the tubes may have reached 30° C.

The behaviour of mixtures with SV concentrates was studied in some detail.

Repeated cycles of combination-elution with the same virus amount.

It is well known (Hirst, 1942) that influenza virus can be adsorbed to and eluted from red cells several times without apparent change of its properties. The experiment below indicates that the interaction between virus and inhibitor of crude allantoic fluids (inhibition→inhibitor destruction) may also be repeated several times by successive addition of fresh normal allantoic fluid, the haemagglutinins being released almost quantitatively each time.

A twofold serial dilution in saline was made of a virus-infected allantoic fluid. Portions of the dilutions were further treated in two ways :

(1) Each virus dilution was mixed with an equal amount of normal allantoic fluid, incubated at room temperature for 30 minutes, then chilled in ice-water, after which the haemagglutination titre of each mixture was determined (Curve A in Fig. 1).

(2) Control, treated in the same way, but with saline substituted for normal allantoic fluid (Curve B in Fig. 1).

Obviously a good deal of the haemagglutination of the mixtures with the lowest concentrations of virus was still inhibited after 30 minutes of incubation at room temperature.

The experiment was repeated in the same way, but with 20 hours of incubation at 35° C. before titration (Fig. 2). After this incubation there existed no signs of inhibition even with the lowest virus concentrations.

New amounts of normal allantoic fluid and saline, respectively, were added to each dilution of the last-mentioned experiment, and the haemagglutination titres were determined after a further incubation at 35° C. for 20 hours (Fig. 3).

There was now a significant drop in the titres of the lowest virus concentrations. However, the control showed the same decrease of titre, suggesting that the smaller maximal " elution " of haemagglutinins in mixtures with low virus concentrations might be only an artefact produced by a more rapid inactivation of haemagglutinins when in these concentrations.

It may be pointed out that Hardy and Horsfall (1948) in similar experiments apparently did not include controls, and they do not mention the possibility of an inactivation of haemagglutinins as a cause of the drop in titre of the low virus concentrations. Moreover, they seem to underestimate the necessity of increasing the incubation time to obtain maximal release of haemagglutinins in mixtures with low concentrations of virus. The validity of their conclusions regarding the existence of " bound virus " in virus-infected allantoic fluid is, therefore, still uncertain.

That the precipitation-dissolution phenomenon can also be repeated with a single virus aliquot by successive additions of fresh inhibitor is shown by the following experiment :

Active SV Concentrate 2 (0·03 ml.) was mixed with the same volume of inhibitor Preparation 1 in a small test-tube. The reagents were kept in ice-water. A precipitate immediately appeared with large floccules adhering to the walls. After being kept in ice-water for 2½ hours the tube was incubated at 35° C. for 15 minutes. The precipitate was dissolved in about 2 minutes. The tube was

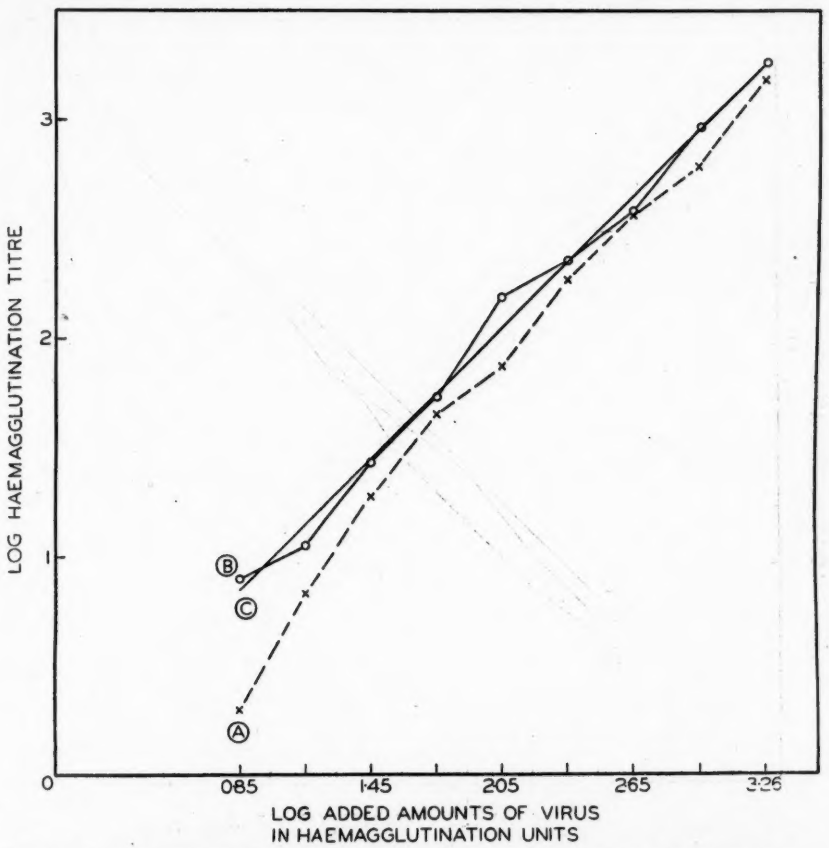

FIG. 1.—Free haemagglutinins in virus—normal allantoic fluid mixtures after incubation for 30 minutes at room temperature. (A) x – – x, Virus diluted in saline + normal allantoic fluid. (B) o——o, Virus diluted in saline + saline ; (C) ——, Haemagglutination titres calculated from control containing highest virus concentration.

again chilled in the ice-water for 10 minutes, the suspension remaining quite clear. After this the whole precipitation-dissolution cycle was repeated three times by adding further aliquots of inhibitor under similar conditions. The reaction ran the same course each time, although the precipitate was a little less flocculent in the last cycles, perhaps depending upon the higher dilution.

Influence of temperature on the dissolution time.

As mentioned above, virus-inhibitor precipitates, formed in the cold, were completely dissolved in a few minutes when brought into a 35° C. incubator. If, on the other hand, the reagents and the mixture were kept at room temperature during the whole operation, only a turbidity appeared immediately and cleared

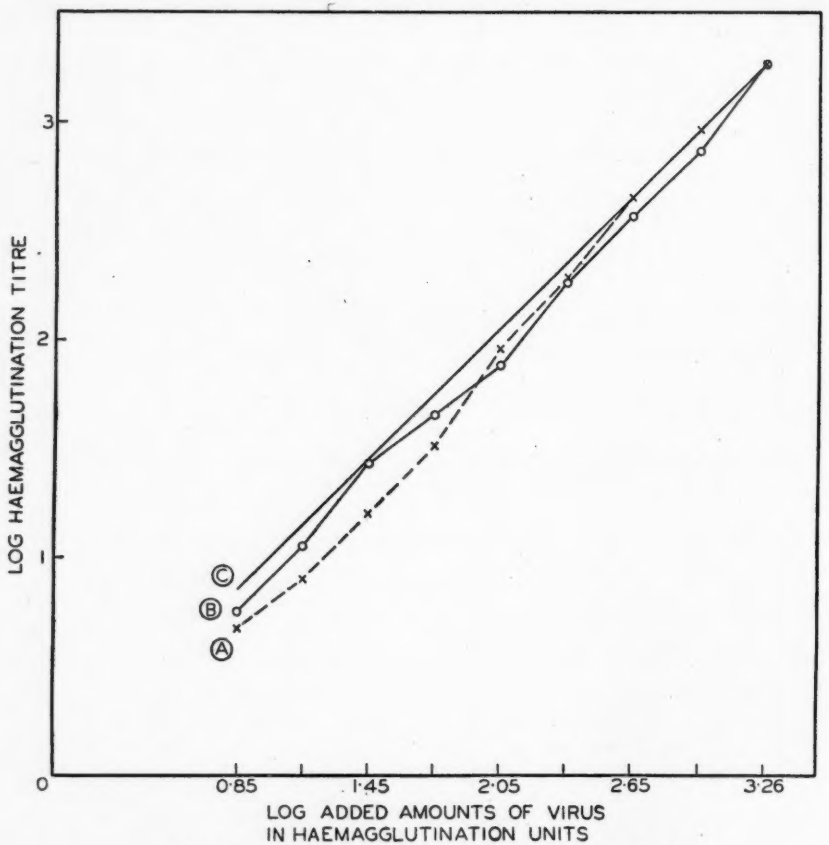

FIG. 2.—As in Fig. 1, but incubation for 20 hours at 35° C.

up in a few moments, showing that the dissolution reaction, at these high concentrations, proceeds very rapidly. At 0 to 1° C., however, even 2½ hours' incubation brought about no visible reduction in the precipitate.

A reaction rate intended to make observation more convenient was established in an experiment performed in a water bath kept at 10·5–11·0° C. Active SV Concentrate 1 (0·15 ml.) was mixed with inhibitor (0·6 ml. Preparation 1) at a temperature of 5° C. A heavy precipitate appeared immediately. The tube

was then kept in the water bath during the whole experiment. It was found that the precipitate was not altered in the first 15 minutes; at 45 minutes a slight reduction was apparent, and after 2 hours and 20 minutes only very slight residues of the floccules could be seen with the magnifying-glass, being completely dissolved a few minutes later (Table IV).

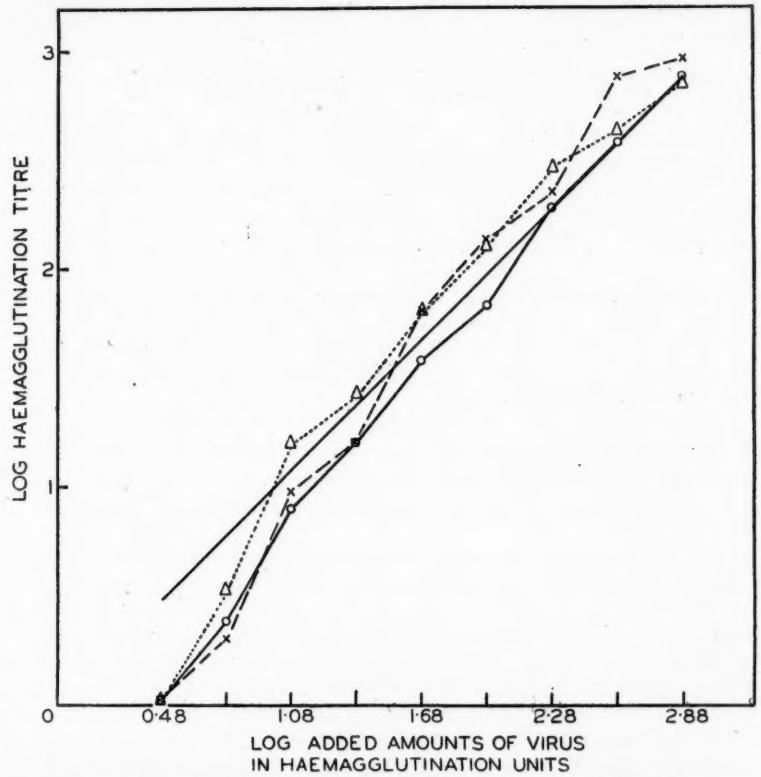

FIG. 3.—Free haemagglutinins in virus—normal allantoic fluid mixtures after incubation for 20 hours at 35° C. with repeat after addition of further allantoic fluid. Δ - - - - Δ, Virus diluted in saline + normal allantoic fluid, incubation for 20 hours at 35° C., repeat after further addition of normal allantoic fluid. x – – x, Virus diluted in saline + saline, incubation for 20 hours at 35° C., repeat after addition of normal allantoic fluid. o——o, Virus diluted in saline + saline, incubation for 20 hours at 35° C., repeat after further addition of saline. ——, Haemagglutination titres calculated from control containing highest virus concentrations.

Correlation between the precipitation-dissolution phenomenon and the release of haemagglutinins.

Some of the precipitation-dissolution experiments described above were also analysed by haemagglutination titrations. 0·1 ml. samples were removed after different periods of time, immediately heated in a water bath at 65° C. for 15

TABLE IV.—*Haemagglutination Titres and the Disappearance of Precipitates on Incubation of Mixtures of 4/5 of the Inhibitor Preparation 1 and 1/5 of the SV Preparation 1.*

Incubation temperature.	Duration of incubation.	Haemagglutination titre of the mixture.*	Approximate gradation of the precipitate as seen through a magnifying-glass.
35° C.	0	4·16	+++†
	3 hrs.	5·26	—
	48 hrs.	5·56	—
10·5°–11·0° C.	0	4·16	+++
	5 min.	4·39	+++
	15 ,,	4·54	+++
	45 ,,	4·69	++
	2 hrs. 20 min.	4·99	(+)
	4 hrs.	5·14	—

* Samples were removed after the indicated periods of time, inactivated in the 65° C. water bath for 15 minutes to stop the process and then kept at 4° C. until titrated.

† When mixed at + 1° C. a heavy precipitate was formed which at 35° C. disappeared in about 3 minutes.

Control; The virus mixed with phosphate buffer instead of inhibitor and then likewise heated at 65°C for 15 minutes. Titre 5·71.

minutes in order to interrupt the process, and then stored at 4° C. until titrated. A virus control with phosphate buffer substituted for inhibitor and treated in the same way was included. Two such experiments with the SV Preparation 1 are listed in Table IV.

It is obvious that the macroscopically visible precipitate had disappeared before the haemagglutination reaction had reached its maximum. The titre, even after 48 hours of incubation, was less than the control, but the difference is not significant. The experiment has been repeated with a similar result. It is, however, impossible to judge from the available data whether the release of virus haemagglutinins from the complex is complete.

The elution of haemagglutinins from a mixture of MV and inhibitor was tested with the same methods. It appeared, however, that these haemagglutinins were almost quantitatively destroyed by the heat treatment that was used to interrupt the elution process, even in the control without inhibitor. Whether this depends on a constant difference in heat resistance between standard virus and modified virus remains to be investigated. It may be mentioned that some older SV concentrates also did not resist the same heat treatment.

However, if titrated without previous heat inactivation the MV-inhibitor mixture reached a titre of 5·31 after incubation at 35° C. for 48 hours, which should be compared with the virus control titre of 5·23, i.e. no significant difference.

SUMMARY.

The interaction between the PR 8 strain of influenza virus and the haemagglutination inhibitor of normal allantoic fluid, both purified and concentrated by two cycles of differential centrifugation, has been studied.

Active virus, which, in this connection, means virus that has retained its capacity to render the inhibitor particles non-inhibitive, immediately gives a visible precipitate when mixed with the inhibitor under suitable conditions. The precipitate is then gradually dissolved, whereby free virus haemagglutinins reappear. The precipitation-dissolution phenomenon can be repeated several times with a single aliquot of virus by successive additions of fresh inhibitor.

The disappearance of the precipitate at these high concentrations proceeds at a fairly rapid rate—at about 30° C. in a few minutes, at 11° C. in about 1 to 2 hours.

From the available results it is not possible to judge whether virus is completely eluted from the virus-inhibitor complex.

Both the classical " standard virus " with a sedimentation constant of about 700 S and the " modified virus " with a sedimentation constant at around 500 S give this precipitation-dissolution phenomenon.

As a whole the precipitation-dissolution reaction reflects the course of the modifying effect of normal allantoic fluid on influenza virus haemagglutination.

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION. PRECIPITATION REACTION IN MIXTURES OF INACTIVE VIRUS AND INHIBITOR.

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THE preceding paper (Svedmyr, 1949) described the formation and dissolution of precipitates in mixtures of purified concentrates of active influenza virus (PR 8) and the inhibitor present in normal allantoic fluid from chick embryos.

In the present paper it will be shown that precipitates obtained with properly pre-heated virus do not dissolve spontaneously. Data regarding the composition of the stable precipitate will be given. Furthermore, the influence of virus-antibodies on the precipitation reaction is described.

MATERIALS AND METHODS.

Heat-inactivated virus preparations.

The virus was inactivated as follows: Flasks containing 100 ml. of the preparations were immersed in a 65° C. water bath for 15 minutes. The temperature of the material exceeded 60° C. for about 10 minutes. The first preparation (heated "standard virus" Preparation 1 = HSV 1) was made from part of the final active preparation SV 1 described in the preceding paper (Svedmyr, 1949). This was diluted 1/50 before heating and afterwards reconcentrated about 50 times by centrifugation in the air-driven centrifuge at 27,000 r.p.m. for 20 minutes. On another occasion (Preparation HSV 2) a fraction of a clarified Sharples concentrate (SV 2) was inactivated and then further purified as previously described for the active virus.

The nitrogen content of the inactive virus concentrates was 3·3 (HSV 1) and 4·8 (HSV 2) mg. per ml.; the haemagglutination titres 6·48 and 6·71 respectively.

Inhibitor preparations.

In addition to the preparations described in the preceding paper (inhibitor Preparations 1 and 2, Svedmyr, 1949) a third concentrate was employed. The starting material was normal allantoic fluid from 14-day-old chick embryos and the purification methods were essentially the same as for Preparation 1. The final concentrate contained 0·75 mg. of nitrogen per ml., and showed an inhibitory activity of 0·83 when titrated in a dilution of 1/1000, as compared with 1·58 for the starting material.

Anti-PR 8 serum.

Serum from a ferret convalescent after infection with PR 8 influenza virus was used. Before use the serum was heated to 56° C. for 30 minutes. The potency of the serum was checked by titration for complement-fixing capacity.

Capillary centrifuge tubes.

For quantitative estimation of the precipitates the reaction mixtures were centrifuged in special tubes, where the sediment was collected in gauged glass capillaries. The volume of the precipitate was estimated from the height of the closely packed sediment. Two sets of tubes were used, one type holding about 0·18 ml., the other, used for diluted preparations, holding 5 ml. The capillary of the second type was graded, that of the former was ungraded, the height of the sediment in that case being determined with calipers. With wooden adapters the tubes could be placed in the buckets of an International Size 3 centrifuge.

The composition of buffer solutions and saline as well as a description of the titration methods is given in the preceding paper (Svedmyr, 1949).

EXPERIMENTAL.

Precipitation with heat-inactivated virus.

Heating to 56° C. for 30 minutes is known to inactivate the "enzymatic" effect of influenza viruses on the red cell receptors (Briody, 1948; Hirst, 1948*b*), as well as on the inhibitors of normal serum (Hirst, 1948*b*; Burnet, 1948) and normal allantoic fluid (Svedmyr, 1948). It was, therefore, to be expected that virus-inhibitor precipitates obtained with heated virus should not show the phenomenon of spontaneous dissolution, provided the virus "enzyme" is responsible for this reaction.

The validity of this assumption was tested in the following experiment:

Heat-inactivated virus (HSV 1) was mixed with inhibitor (Preparation 1) in the proportions 1 to 5 at room temperature. A flocculent precipitate appeared, remaining unchanged during a further incubation at 35° C. for 48 hours. Titrations for haemagglutinins showed no difference between samples taken immediately after mixing, after 3 and after 48 hours at 35° C. The titres were 1·68 as compared with the virus control 5·71, indicating that no virus was eluted during incubation.

Quantitative data on the virus-inhibitor precipitation.

The formation of a stable precipitate in mixtures of HSV and inhibitor made it possible to collect quantitative data regarding the precipitation reaction. An attempt was made to study to what extent the amount of precipitate and the activity of the supernatant were dependent upon the concentrations of the reactants.

In the special centrifuge tubes mixtures were prepared of constant amounts of one preparation with aliquots of serial dilutions of the other, measured by means of capillary pipettes or a microburette. A control containing only virus and phosphate buffer was included in each series. The precipitates were observed under a magnifying-glass and their appearance recorded, after which the tubes were left at room temperature for about 4 hours; or, in the first experiment, for

30 minutes and then at 4° overnight. The tubes were then spun in a bucket type centrifuge. The sediments were measured and the supernatants removed for haemagglutination tests.

It appeared that in high concentrations the sedimentation of the precipitate was delayed in tubes with excess of one of the components, as could be expected from the high viscosity of the preparations: the greater the excess, the slower the sedimentation. Therefore, after removal of the supernatants the sediments were further centrifuged until the volumes appeared constant. In the last experiment the sedimentation disturbances caused by viscosity variations were minimized by diluting the mixture 1/100 in phosphate buffer before centrifugation.

The results of two typical experiments are indicated in Tables I and II and Fig. 1 and 2.

TABLE I.—*Variation in the Virus-inhibitor Precipitate with Varying Concentrations of Inhibitor. Constant Quantity of Virus: HSV 2–0·02 mg. Nitrogen per Tube.*

Added amount of inhitor Preparation 2 in mg. nitrogen.	Approximate gradation of the immediate flocculation.	Height in mm. of the sediment after centrifugation for 3½ hr. at 1700 r.p.m. and 1 hr. at 2400 r.p.m.	Haemagglutination titre of the supernatant after 1½ hr. at 1700 r.p.m.
0·041	+++	6·0	<2·09
		still not homogeneous	
0·041/2	+++	4·4	<1·89
		still not homogeneous	
0·041/4	+++	3·7	<1·89
0·041/8	+++	2·9	<1·89
0·041/16	+++	2·2	1·89
0·041/32	+++	1·5	2·64
0·041/64	+++	1·4	3·69
0·041/128	++	1·1	4·14
0·041/256	(+)	0·9	4·52
0·041/512	—	0·9	4·67
0	—	..	4·82

TABLE II.—*Variation in the Virus-inhibitor Precipitate with Varying Concentrations of Virus. Constant Quantity of Inhibitor Preparation* 3–0·0038 *mg. Nitrogen per Tube, except the Control. Before Centrifugation the Mixture was Diluted* 1/100 *in Phosphate Buffer.*

Added amount of HSV 2 in mg. nitrogen.	Approximate gradation of the immediate flocculation.	Volume of the sediment in mm.³ after 4½ hr. at 2800 r.p.m.	Hemagglutination titre of the supernatant after 30 min. at 2800 r.p.m.
0·17	+++	6·0	3·16
0·17/2	+++	2·9	2·56
0·17/4	+++	1·3	1·28
0·17/8	++	0·6	0·30
0·17/16	+	*ca* 0·3	<0·30
0·17/32	—	*ca* 0·1	<0·30
0·17, no inhibitor	—	2·0	3·91

An experiment in which decreasing amounts of inhibitor were added to constant amounts of virus (Table I, Fig. 1) showed that haemagglutinins appeared in the supernatant when the original concentration of inhibitor was below a certain level. A closer analysis reveals that the amount of virus fixed in the

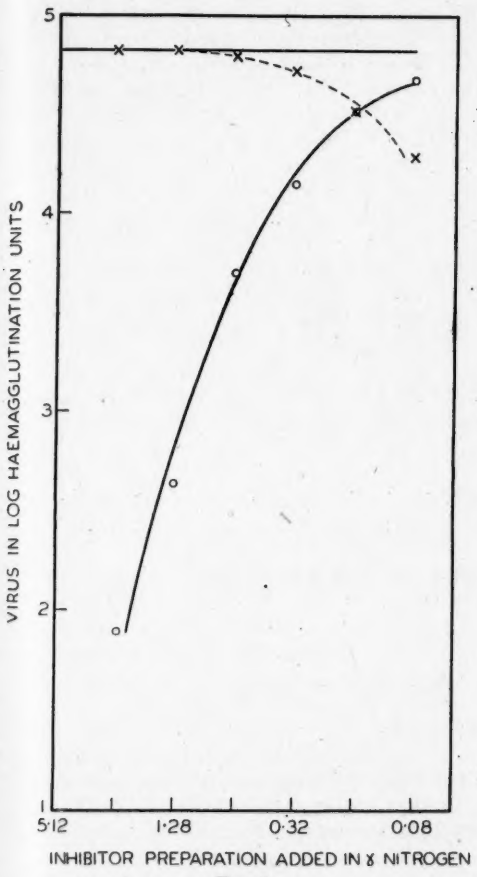

FIG. 1.

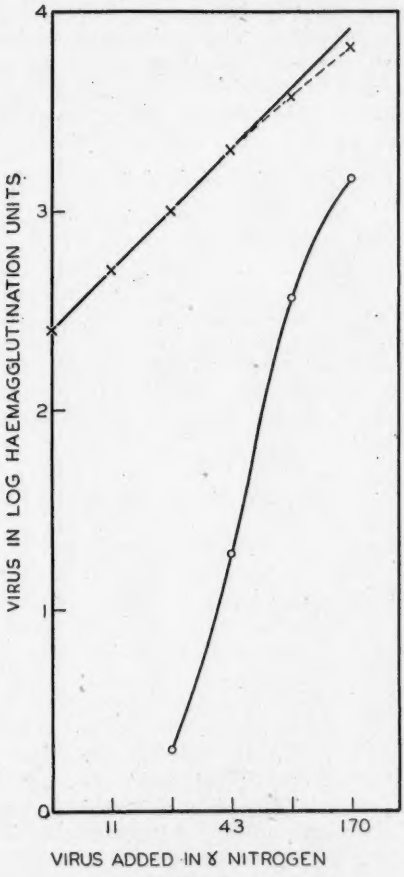

FIG. 2.

FIG. 1.—Variations of free and bound virus in mixtures with varying concentrations of inhibitor. o——o = Free virus ; x - - - x = bound virus ; —— = added virus.

FIG. 2.—Variations of free and bound virus in mixtures with varying concentrations of virus. o——o = Free virus ; x - - - x = bound virus ; —— = added virus.

precipitate is not proportionate to the quantity of inhibitor. Under the given conditions proportionately more virus is bound the smaller the amount of inhibitor used. The results of haemagglutinin titration and measurement of precipitate volume coincide largely in this respect. However, by direct observation of the

newly formed precipitate it was impossible to detect significant differences in the appearance of the precipitate, except in tubes with very small amounts of inhibitor. Direct observation, therefore, cannot be relied upon for quantitative estimations.

The results recorded in Table II and Fig. 2 are in keeping with those of the previous experiment. The more virus is added to a certain amount of inhibitor the larger is the quantity incorporated in the precipitate.

The figures in Table I seem to indicate that the reverse may also be true. If so, the inhibitor, when added in excess, seems to be less readily incorporated. Further experiments, including titrations for inhibitory activity, are necessary before this question can be answered.

Inhibition of precipitation reaction by specific PR 8 antiserum.

The effect of anti-serum against PR 8 virus on the virus-inhibitor precipitation was tested in the following way :

PR 8 ferret serum (0·20 ml.) was treated with 0·05 ml. of M/10 $NaIO_4$ (final dilution M/50) at room temperature for 2·5 hours in order to eliminate the effect of the normal serum inhibitor (Hirst, 1948*a*). The excess $NaIO_4$ was then destroyed by adding 0·05 ml. of 10 per cent glucose.

Three tests were performed :

(1) 0·01 ml. of heat-inactivated standard virus was mixed with 0·09 ml. of the periodate-treated serum and kept at room temperature for 30 minutes. The mixture was clear. After that 0·04 ml. of inhibitor was added. No precipitate appeared.

(2) Inhibitor and virus were mixed first, the typical flocculent precipitate appearing. After 30 minutes at room temperature the serum was added. The precipitate remained unchanged.

(3) Virus was diluted with phosphate buffer instead of serum, inhibitor added last. A typical precipitate appeared.

The three tubes were then incubated at 35° C. After one day a very slight deposit appeared in the first tube, while in Tubes 2 and 3 the precipitates had settled into heavy sediments, the supernatants remaining rather clear. The appearance was quite the same throughout 4 days of incubation.

Obviously the immune serum could prevent the virus-inhibitor precipitation even if it were added in a concentration forming only insignificant amounts of immuno-precipitate when mixed with the virus. However, the same amount of virus-antibody was not able to displace the inhibitor in the combination with virus, at any rate not enough to dissolve the precipitate.

DISCUSSION.

The results reported above clearly indicate that the composition of the virus-inhibitor precipitate varies with the original concentrations of the components. On the basis of available data a calculation of the composition of the precipitate was attempted.

The ratio of inhibitor to virus particles may be computed as follows :

$$\frac{\text{Number of inhibitor particles}}{\text{Number of virus particles}} = \frac{N_i \,.\, k \,.\, s_v^{\frac{3}{2}}}{N_v \,.\, s_i^{\frac{3}{2}}} \cdot \frac{(1 - F_i)}{(1 - F_v)},$$

where :

N_i = amount of inhibitor preparation nitrogen added,

N_v = amount of virus nitrogen added,

k = correction factor for impurities (" 30 S ") present in the inhibitor preparation. It was calculated from ultracentrifugation data.

s_i = the sedimentation constant of the inhibitor particles (= 200 S),

s_v = the sedimentation constant of the standard virus particles (=700 S),

F_i = fraction of total inhibitor remaining uncombined,

F_v = fraction of total virus remaining uncombined.

The formula, of course, involves many approximations. Some should be discussed.

The amount of material is measured in terms of the nitrogen content. This is permissible on the condition that virus and inhibitor have the same nitrogen content. According to Knight (1944) this should be the case. Furthermore, the impurities (" 30 S " particles) should have the same nitrogen content. On this point no information is available at present.

The particle weight is assumed proportional to the 3/2 power of the sedimentation constant. This presupposes a spherical shape of the particles and identity of partial specific volumes, about which information is insufficient.

The formula given may, however, at least give an idea of the order of magnitude of the ratio mentioned.

From the data in the two experiments related above the ratios 1/1·2 and 1/0·9 were computed for mixtures containing just enough inhibitor to precipitate the total virus amount added, and another experiment, not listed above (constant inhibitor versus varying virus amounts, mixtures not diluted before centrifugation) yielded the ratio 1/1·8. The values from different experiments thus appear in good agreement, indicating that about one virus particle is inhibited by one inhibitor particle. The largest proportion of virus particles incorporated in the precipitate can be calculated from the values in Fig. 2. In the highest inhibitor dilution the precipitate was probably saturated with virus particles. Here about 70 virus particles were added per inhibitor particle, and as about 2/7 were fixed in the precipitate, this should contain about 20 virus particles per inhibitor particle—a rather surprising proportion of the large virus particles to the much small inhibitor particles.

The observation that virus-antibodies prevent the precipitation of virus with inhibitor is in keeping with the inhibition of haemagglutination by specific antibodies. These reactions indicate that the antibody somehow blocks the chemical structures on the virus particle that combine with the inhibitor. This, of course, does not necessarily imply that the antibody and the receptor groups of the inhibitor are chemical analogues.

SUMMARY.

Virus, the inhibitor-destroying capacity of which has been inactivated by heat treatment, gives a precipitate with inhibitor which does not dissolve spontaneously on incubation. The composition of this precipitate varies with the proportions of the components added,

Virus antibodies prevent the precipitation when added to the virus before the inhibitor is added.

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STUDIES ON A FACTOR IN NORMAL ALLANTOIC FLUID INHIBITING INFLUENZA VIRUS HAEMAGGLUTINATION. THE EFFECT OF ACTIVE VIRUS, PROTEOLYTIC ENZYMES AND PERIODATE ON THE INHIBITOR.

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IN the two preceding papers (Svedmyr, 1949*a*, 1949*b*) a description was given of the precipitation-dissolution reaction between purified concentrates of the PR 8 strain of influenza virus and the inhibitor present in normal allantoic fluid from chick embryos.

In this paper some observations are reported regarding the effect on the inhibitor of treatment with active virus, proteolytic enzymes or periodate, as well as the action of virus and periodate upon a stable virus-inhibitor precipitate.

MATERIALS AND METHODS.

The virus and inhibitor preparations as well as the buffer and saline solutions used were described in the preceding publications (Svedmyr, 1949*a*, 1949*b*).

Crystalline trypsin and crystalline soybean trypsin inhibitor (Kunitz, 1946) were obtained through the courtesy of Dr. Moses Kunitz, of the Rockefeller Institute for Medical Research.

The trypsin preparation, containing about 50 per cent. $MgSo_4$, was dissolved in saline and then carefully washed on the ultrafilter (Seibert, 1928) with several lots of saline to remove the $MgSO_4$, and then with phosphate buffer containing 100 units each of penicillin and streptomycin per ml. as preservative. Finally, the stock solution was made up to contain about 10 mg. trypsin per ml. The ultrafiltration was carried out at 2° C.

The crystalline soybean trypsin inhibitor was kept in stock solution at a concentration of 10 mg. per ml. of phosphate buffer with preservatives as mentioned above.

Ficin was used as a commercial preparation, also stored in a stock solution of 10 mg. per ml. phosphate buffer with the same preservatives.

The activity of the trypsin and ficin solutions was tested on clots of human fibrin.

All stock suspensions of virus, inhibitor and enzymes were stored at about 4° C.

Chemically pure, crystalline $NaIO_4$ was dissolved to a concentration of M/10 in saline and kept as stock solution. Further dilutions were always made in saline and are expressed throughout in the final molarity of $NaIO_4$.

A 10 per cent solution of analytically pure glucose in phosphate buffer was used as periodate-inactivator with the same preservatives as mentioned above.

Haemagglutination tests were usually performed as described previously (Svedmyr, 1948*a*). In several experiments, however, in addition to active virus, heat-inactivated virus (kept in the water bath at 65° C. for 15 minutes) was used for the titrations of inhibitory activity. If so, the virus-inhibitor mixture was usually kept at room temperature for one hour before the addition of red cells.

Some other modifications are mentioned in the text.

EXPERIMENTAL.

The Effect of Small Amounts of Active Virus on the Inhibitor.

In a previous paper (Svedmyr, 1948*b*) it was shown that active virus, even in small quantities, has the capacity to gradually destroy the inhibitory activity of normal allantoic fluid. After sufficient destruction or modification of the inhibitor, haemagglutinins appear in the previously non-haemagglutinating mixture. The inhibitor-destroying capacity of the virus was eliminated by a suitable heat treatment.

The following experiment was designed to study the influence of small amounts of virus on purified inhibitor concentrates, and test the results by titrations for residual inhibitory activity and by precipitation with heat-inactivated virus concentrates.

Preparations of active and heat-inactivated "standard virus" (SV) and of "modified virus" (MV) were diluted 1/100 in phosphate buffer. Each dilution was mixed with inhibitor in the proportions 1 + 4, making a final concentration of 1/500 of the virus concentrate. A control with phosphate buffer substituted for the virus was included. The mixtures were incubated for 48 hours at 35° C. Samples were removed after different periods of time, heated in the 65° C. water bath for 15 minutes in order to interrupt the elution process, and then stored at 4° C. until titrated for residual inhibitory activity. After 48 hours of incubation a sample was also tested for capacity to precipitate with heat-inactivated virus. The results are listed in Table I.

It is clear that even relatively small amounts of active virus gradually destroy both the inhibitory and the precipitating capacity of the inhibitor. The heat-inactivated virus had lost all such activity.

TABLE I.—*The Effect of Active and Inactive Virus Preparations in the Final Dilution 1/500 on the Inhibitor.*

Virus material.	Duration of incubation at 35° C.	Active SV 1/500.	Active MV 1/500.	Heat-inactivated SV 1/500.	Control without virus.
Residual inhibitory activity of the inhibitor preparation incubated with virus*	0	0·75	0·98	0·67	0·82
	3 hr.	0·22	0·38	0·75	—
	18 hr.	0·22	..	—	0·82
	48 hr.	0·22†	0	0·75	0·82
Precipitation with 1/5 volume of heat-inactivated SV (test sample incubated for 48 hrs.)		—	—	+	+

* Samples were removed after the indicated periods of time and heated at 65° C. for 15 minutes to interrupt the process. The series with MV was then diluted in saline 1/160 and titrated directly; the other series were diluted 1/200, and all samples, except those with heat-inactivated virus, boiled before titration.

† Positive agglutination before boiling.

The Effect of Proteolytic Enzymes on the Inhibitor.

Crystalline trypsin.

Several authors have reported that crystalline trypsin destroys influenza virus receptors of red cells (Hirst, 1948), as well as the inhibitor of normal serum (Hirst, 1948; McCrea, 1948) and that of normal allantoic fluid (Hardy and Horsfall, 1948).

In a series of preliminary experiments with crude allantoic fluids it was found difficult to evaluate the results owing to the haemagglutinating effect of the trypsin itself. As previously shown by Volkert and Horsfall (1947) in experiments on pneumonia virus of mice, this difficulty can be overcome by addition of crystalline soybean trypsin inhibitor (Kunitz, 1946). Consequently the trypsin inhibitor was used in the present experiments. In the concentrations employed it did not interfere with virus haemagglutination titres or with virus-inhibitor precipitation tests.

Effect of varying concentrations.—First, the influence of varying concentrations of trypsin on the inhibitor was studied. One volume of trypsin dilution was added to four volumes of the inhibitor, the final concentrations of trypsin being 2, 0·5, 0·1 and 0·02 mg. per ml. The mixtures were incubated at 35° C. for three hours, after which trypsin inhibitor was added in excess. The mixtures were then tested for capacity to precipitate with heat-inactivated virus, and titrated for residual inhibitory activity in the usual way (Table II).

Crystalline trypsin, even in a concentration of 2 mg. per ml., failed to destroy the capacity of the inhibitor to precipitate with heat-inactivated virus. The inhibitory activity, however, was reduced, although not completely destroyed. In this time interval about 0·1 mg. of trypsin per ml. was needed for a significant effect.

Control tests showed that no precipitate was formed in mixtures of trypsin (2 mg. per ml.) and virus without inhibitor.

Effect of length of incubation.—The influence of the length of incubation on the trypsin effect was tested in a similar experiment with the use of 0·1 mg. of trypsin

TABLE II.—*The Effect of Varying Concentrations of Crystalline Trypsin on the Inhibitor by Incubation at* 35° C. *for* 3 *Hours.*

Concentration of trypsin.	Residual inhibitory activity* against active virus.	Residual inhibitory activity* against inactivated (65° C. for 15 min.) virus.
2 mg./ml.	0·15	2·05
0·5 mg./ml.	0·37	2·05
0·1 mg./ml.	0·60	2·20
0·02 mg./ml.	1·35	2·65
0	1·65	2·80

* Determined on a 1/150 dilution of the inhibitor material.

per ml. Samples were removed after different periods of time, and the trypsin inactivated by addition of excess trypsin inhibitor (Table III).

TABLE III.—*Residual Inhibitory Activity of an Inhibitor Preparation after Treatment with* 0·1 *mg. of Crystalline Trypsin per ml. for Various Periods of Time.*

Duration of incubation.	Residual inhibitory activity of the material treated with trypsin.*	Control : Trypsin already inactivated by trypsin-inhibitor and added to the preparation after incubation.
0	1·36	..
30 min.	0·83	..
3 hr.	0·38+	1·36
15 hr.	0·23+	..

* Determined on a 1/160 dilution of the inhibitor material.
\+ = positive precipitation reaction with heat-inactivated SV.

Apparently the inhibitory activity decreased gradually, but it was not completely lost even after 15 hours. The final product also retained the capacity to precipitate with virus. The latter reaction was tested with heat-inactivated virus as well as with active virus in the cold. In both cases heavy precipitates appeared, that with active virus being dissolved in the typical way within a few minutes if kept at 35° C. (Svedmyr, 1949*a*).

Control tests showed that increase of the concentration of trypsin to 2 mg. per ml. did not cause any further reduction of the inhibitory activity after 17 hours at 35° C.

In one experiment the residual amount of inhibitor was assayed with two different methods. The inhibitor was treated with 0·4 mg. of trypsin per ml. at 35° C. for 20 hours, after which an excess of trypsin inhibitor was added. The reaction mixtures were then titrated for inhibitory activity in two principally different ways, in both cases against active as well as heat-inactivated purified virus. As in other experiments a control was included—inhibitor incubated without trypsin and then mixed with trypsin already inactivated.

1. The virus was serially diluted in 0·25 ml. amounts of saline, 0·25 ml. of the test material diluted 1/100 in saline, and finally 0·5 ml. of red cell suspension were added. The mixtures of heat-inactivated virus and inhibitor were kept at room temperature for 1 hour before the addition of red cells.

2. The test material was serially diluted in 0·25 ml. amounts of saline, 0·25 ml. containing 8 agglutinating doses of virus, and finally 0·5 ml. of red cells were added. Mixtures of inactive virus and inhibitor were likewise kept at room temperature for 1 hour before the addition of red cells.

The results are listed in Table IV.

TABLE IV.—*Residual Inhibitory Activity* after Trypsin Treatment of the Inhibitor as Titrated with Different Methods.*

Titration method.	Residual inhibitory activity against active virus.		Residual inhibitory activity against heat-inactivated virus.	
	Material treated with trypsin.	Control without trypsin treatment.	Material treated with trypsin.	Control without trypsin treatment.
I	0·38	1·73	2·03	2·93
II	<1·00	1·83	1·98	2·73

* Determined on a 1/100 dilution of the inhibitor material. Active and heat-inactivated SV were used as test viruses.

The results show that 5–25 per cent of the inhibitory activity resisted trypsin treatment. As can be seen from data in the preceding paper (Svedmyr, 1949*b*) a corresponding reduction of the inhibitor concentration will not have any appreciable influence upon the precipitation reaction.

Ficin.

An attempt was also made to destroy the precipitative capacity of the inhibitor by means of a commercial preparation of ficin. It was found, however, that incubation at 35° C. for 2½ hours with 2 mg. ficin per ml. did not destroy the capacity of inhibitor to precipitate with heat-inactivated virus. Further incubation at 35° C. for two days in the presence of ficin did not change the appearance of the precipitate. A control revealed that practically no precipitate was formed in mixtures of virus and ficin without inhibitor.

The Effect of Periodate on the Inhibitor.

Hirst (1945, 1948) described the destruction of red cell receptors and the inhibitor of normal rabbit serum by rather low concentrations of sodium periodate as opposed to other oxidants. It has been shown that periodate has the same effect on virus inhibitors from other sources, such as mucins of various human origins (Anderson, Burnet, Fazekas, McCrea and Stone, 1948) and also the inhibitor of normal allantoic fluid (Hardy and Horsfall, 1948). The action of periodate has been taken as an evidence that carbohydrate is an essential part of the receptors, since it is known that periodate in biological materials chiefly attacks carbohydrates. This assumption has later been very much corroborated by the isolation from red cells of very potent inhibiting substances containing a high percentage of carbohydrate (de Burgh, Yu, Howe and Bovarnick, 1948; Woolley, 1949).

The effect of sodium periodate on the purified inhibitor concentrates from normal allantoic fluid was studied in some experiments.

Effect of different concentrations.

First, the effect of different concentrations of periodate on the inhibitory activity was tested. Twofold serial dilutions of $NaIO_4$ were made in saline, and each dilution in an amount of 0·04 ml. was mixed with 0·16 ml. of inhibitor. The final concentrations of periodate covered the range of M/100 to M/1600. After two hours at room temperature 0·10 ml. of 10 per cent glucose was added to each sample to destroy the excess of periodate, and after dilution with 32 ml. of saline, i.e. 200 times the original volume of inhibitor preparation, each mixture was titrated for residual inhibitory activity against both active and heat-inactivated virus. For results see Table V.

TABLE V.—*The Effect of Different Concentrations of Periodate on the Inhibitory Activity.*

Concentration of $NaIO_4$.	Residual inhibitory activity against active virus.	Residual inhibitory activity against inactivated (65° C. for 15 min.) virus.
M/100	0·23	0·03
M/200	0·15	0·10
M/400	0·23	0·33
M/800	0·38	0·33
M/1600	1·66	2·51
0	1·58	2·73

Concentrations of periodate down to M/800 eliminated the inhibitory activity, the titre values shown being insignificant even against inactive virus. At a dilution of M/1600, however, the periodate failed to influence the inhibitory activity.

In another experiment, now with periodate in the concentration of M/150, samples were removed from the inhibitor-periodate mixture after different time intervals at room temperature and the excess periodate immediately destroyed by glucose. It appeared (Table VI) that the inhibitory capacity decreased precipitately, and the final product had lost its capacity to precipitate with heat-inactivated virus.

TABLE VI.—*The Effect of Periodate* (M/150) *on the Inhibitor as Tested after Different Periods of Time.*

Duration of incubation at room temperature.	Inhibitor treated with periodate M/150.		Inhibitor treated with a mixture of periodate and glucose.	Control: Inhibitor without periodate.	
	Residual inhibitory activity.	Precipitation reaction with heat-inactivated virus.	Residual inhibitory activity.	Residual inhibitory activity.	Precipitation reaction with heat-inactivated virus.
About 30 sec.	0·45	..	..	..	..
5 min.	0	..	..	..	..
2 hr.	0	Negative	1·36	1·43	Positive

The titrations for inhibitory activity were performed on a 1/160 dilution of the inhibitor material.

Effect on precipitation dissolution reaction.—The effect of different concentrations of periodate on the precipitation-dissolution reaction of the inhibitor with active virus was tested in some experiments. Various dilutions of periodate (0·01 ml.) were added to 0·03 ml. amounts of inhibitor. The mixtures were kept for at least 2 hours at room temperature, after which 0·01 ml. of 10 per cent glucose was added to each tube to destroy excess periodate. The tubes were then placed in ice-water with subsequent addition of 0·01 ml. of active standard virus or 0·05 ml. of active modified virus. Both virus preparations and pipettes were pre-chilled. The appearance of precipitates was recorded, and the tubes left for at least 30 minutes in the ice-water. They were then incubated at 35° C. or at room temperature and the approximate dissolution times were noted (Tables VII–X).

The results reveal that with the higher concentrations of periodate, down to about M/600, the precipitation capacity of the inhibitor was destroyed, and that the lower concentrations of periodate did not influence the formation of a precipitate. This is in keeping with the above-mentioned effect of various periodate concentrations on the inhibitory activity. Within a rather narrow zone of periodate concentration, however, M/1440 and M/1350 respectively in two experiments, the dissolution reaction of precipitates with active SV was significantly modified

TABLE VII.—*Precipitation-dissolution Reaction Between Active SV and Periodate-treated Inhibitor.*

Periodate concentrations.	The appearance of the precipitates after the indicated periods of time.					
	Immediately at 1° C.	30–60 min. at 1° C.	2 min. at 35° C.	5 min. at 35° C.	15 min. at 35° C.	30 min. at 35° C.	Overnight 35° C.
M/160	−	−	−	−	−	−	−
M/180	−	−	−	−	−	−	−
M/1440	+++	+++	++(+)	++(+)	++	+(+)	−
M/4320	+++	+++	++(+)	++	−	−	−
M/12,960	+++	+++	++(+)	++	−	−	−
M/38,880	+++	++(+)	++(+)	++	−	−	−
M/116,640	+++	+++	++(+)	++	−	−	−
Control: inhibitor treated with a mixture of periodate and glucose	+++	+++	++(+)	++	−	−	−
Untreated inhibitor	+++	+++	++(+)	++	−	−	−

TABLE VIII.—*Experiment as in Table VII. Shorter Intervals Between the Periodate Concentrations.*

Periodate concentrations	The appearances of the precipitates after the indicated periods of time.									
	Immediately at 1° C.	30–55 min. at 1° C.	5 min. at 35° C.	10 min. at 35° C.	15 min. at 35° C.	20 min. at 35° C.	25 min. at 35° C.	30 min. at 35° C.	35 min. at 35° C.	Overnight at 35° C.
M/400	−	−	−	−	−	−				−
M/600	+	+	−	−	−	−				−
M/900	+++	+++	++	−	−	−				−
M/1350	+++	+++	++	++	+(+)	+	+	(+)	−	−
M/2025	+++	+++	++	+	−	−				−
M/3040	+++	+++	++	−	−	−				−
M/4560	+++	+++	++	−	−	−				−
M/6840	+++	+++	++	−	−	−				−
Untreated inhibitor	+++	+++	++	−	−	−				−

TABLE IX.—*Precipitation-dissolution Reaction Between Active MV and Periodate-Treated Inhibitor.*

Periodate concentrations.	The appearance of the precipitates after the indicated periods of time.				
	Immediately at 1° C.	30–60 min. at 1° C.	5 min. at 35° C.	10 min. at 35° C.	Overnight at 35° C.
M/400	−	−	−	−	−
M/600	−	−	−	−	−
M/900	+++	+++	+	−	−
M/1350	+++	+++	+	−	−
M/2025	+++	+++	+	−	−
M/3040	+++	+++	+	−	−
M/4560	+++	+++	+	−	−
M/6840	+++	+++	(+)	−	−
Untreated inhibitor	+++	+++	(+)	−	−

TABLE X.—*Experiment as in Table IX. Shorter Intervals Between the Periodate Concentrations.*

Periodate concentrations.	The appearance of the precipitates after the indicated periods of time.					
	Immediately at 1° C.	30–60 min. at 1° C.	5 min. at room temp.	10 min. at room temp.	15 min. at room temp.	Overnight room temp.
M/600	−	−	−	−	−	−
M/900	++	+	−	−	−	−
M/1080	++	+++	++	(+)	−	−
M/1200	++	+++	+	(+)	−	−
M/1350	++	+++	++	(+)	−	−
M/1620	++	+++	++	(+)	−	−
M/1800	++	+++	++	(+)	−	−
M/2025	++	+++	++	(+)	−	−
M/3040	++	+++	++	(+)	−	−
Untreated inhibitor	++	+++	+(+)	(+)	−	−

inasmuch as the reaction time was prolonged. It is striking that in one experiment there was a concentration of periodate intermediate between the "destructive" and "modifying" doses that apparently did not influence the course of the precipitation-dissolution reaction. This might, however, be an artefact, for instance the effect of accidental reduction of the periodate by threads of cotton-wool.

The phenomenon of a prolonged dissolution time was not observed in precipitates containing active modified virus, although (*a*) rigid precautions were taken to avoid an accidental inactivation of the periodate, and (*b*) the intervals between the periodate concentrations were made very short.

It was shown above that both active virus and suitable concentrations of periodate destroy the inhibitory activity of the inhibitor as well as their capacity to precipitate with virus. These agents were also tested for their capacity to release heat-inactivated virus particles from a fresh stable precipitate with inhibitor.

In each of two tubes 0·20 ml. of inhibitor was mixed with 0·05 ml. of heat-inactivated standard virus. The mixtures were kept at room temperature for one hour and the precipitates were then sedimented at 3500 r.p.m. for 20 minutes in an angle centrifuge. The heavy sediments were treated in two ways:

1. One sediment was resuspended in 0·25 ml. of M/100 $NaIO_4$ at room temperature. After a few minutes the precipitate appeared dissolved. The mixture was left at 4° C. overnight and then for two hours at room temperature before

the addition of 0·25 ml. of a 10 per cent solution of glucose to destroy excess periodate. It was then again centrifuged at 3500 r.p.m. for 20 minutes, and the rather slight sediment resuspended in 0·25 ml. phosphate buffer.

2. The other sediment was resuspended in 0·25 ml. of a dilution of 1/100 in phosphate buffer of active standard virus. Together with a control tube with the same dilution of active virus it was then incubated at 35° C. overnight. The mixture still showed a large sediment which, after a final centrifugation at 3500 r.p.m. for 20 minutes, was resuspended in 0·25 ml. of phosphate buffer.

The various fractions and controls, including the original heat-inactivated virus, were then titrated for haemagglutinins. The results are shown in Table XI. All titres are corrected for volume changes, so that the values are directly comparable.

TABLE XI.—*Treatment of the Virus Inhibitor Precipitate with Active Virus and Periodate.*

	Tested material.	Haemagglutination titre.
I.	Supernatant of the mixture before treatment . . .	2·36
	,, ,, after periodate treatment . .	5·44
	Sediment ,, ,, . .	4·31
II.	Supernatant of the mixture before treatment . . .	2·36
	,, ,, after treatment with active virus	5·06
	Sediment ,, ,, ,,	4·24
	Control titre of the heat-inactivated virus	5·63
	,, ,, active virus	4·16

Treatment with periodate obviously released roughly 2/3 of the haemagglutinins, and only a small fraction of the original precipitate could be recovered by centrifugation. Active virus left the bulk of the precipitate unchanged, and only about 1/4 of the haemagglutinins were released. It may be mentioned, however, that in another experiment with a precipitate several weeks old much less haemagglutinin was released whether periodate or active virus were used. On the other hand, in a similar control experiment a fresh precipitate of inactive virus and inhibitor was not visibly altered when incubated at 35° C. for one week in a suspension containing active virus.

It may be mentioned that previous trypsin treatment of the inhibitor component of the precipitate did not influence the capacity of periodate to dissolve the precipitate. Thus the floccules formed in the experiment listed in Table II were dissolved in a few minutes after addition of $NaIO_4$ in a final concentration of M/150. The same concentration of periodate destroyed the capacity of a trypsin-treated inhibitor preparation (2 mg. trypsin per ml. 35° C. overnight) to precipitate with heat-inactivated virus.

DISCUSSION.

Hirst (1942) suggested that the adsorption and elution of influenza virus to red cells was due to the interaction between an enzyme incorporated in the virus particle and its substrate, the receptor, on the red cell surface. This hypothesis was later adapted for the similar adsorption-elution of influenza virus to the cells

in the excised mouse lung (Hirst, 1943). The inhibiting effect of normal serum (Francis, 1947 ; Hirst, 1948 ; Anderson, 1948) and of mucins of various origins (Anderson *et al.*, 1948), as well as the destruction of this effect on incubation with virus, has been explained on the same basis.

The evidence for this enzyme theory is twofold ; the reaction can be repeated many times with the same aliquot of virus without reduction of virus activity ; furthermore, the elution process may be prevented by previous heating of the virus to 56° C. for 30 minutes.

As yet little is known about the chemical structure of the receptors of different origins, and nothing about the change in this configuration following the action of the presumptive virus enzyme. A considerable amount of data has accumulated that seems to indicate that the different receptors are mucoproteins, as indicated by the sensitivity to periodate and trypsin (Hirst, 1945, 1948 ; Anderson *et al.*, 1948 ; Hardy and Horsfall, 1948), by the high inhibitory activity of crude mucinous suspensions, and of purified mucins of different human origins (Anderson *et al.*, 1948), and above all by the preparation from red cells of very active inhibitors containing a large percentage of carbohydrate (de Burgh *et al.*, 1948 ; Woolley, 1949).

The biological implication of the presumptive viral enzyme has been postulated first by Hirst (1943) and later by Burnet (1948*a*), who suggested that the initial stage of influenza virus infection was the adsorption of virus to receptors on the cell surface. After enzymatic modification of the receptor, the virus was then ready for the next step in the infection. The significance of an adsorption of virus to the cell receptors seems established by the prophylactic effect of the " receptor-destroying enzyme " against influenza virus infection in eggs and mice (Stone, 1948*a*, *b*). For the next step in the infection Fazekas and Graham (1949), however, very recently formulated a new hypothesis. Fazekas (1949) showed that moderate doses of periodate modified the receptors of red cells and of cells lining the allantoic cavity of chick embryos. He stated that virus adsorbed to modified cells could not elute, and as he was also able to show that virus could infect the allantoic cavity even if the receptors were modified, he concluded that the viral enzyme action on the receptors is not an obligate phase in the initial stages of influenza virus infection.

The inhibitor of the influenza virus haemagglutination present in normal allantoic fluid has been described by the author in previous publications. The bulk of the results are in keeping with the observations regarding other virus receptors as summarized above.

This inhibitor has been shown to be a particulate component that appears in the allantoic fluid in increasing amounts after the 7th day of incubation. It seems, thus, that it is a product of cell activity, probably released cell receptors.

It is of particular interest that the interaction between virus and inhibitor manifests itself macroscopically as a precipitation reaction, the combination phase, followed by dissolution of the precipitate, the elution phase. It is remarkable that the precipitate, as studied in the stable form with inactive virus, varies in composition within wide limits, depending on the proportions of added ingredients.

The observation that the inhibitor-destroying capacity follows the other activities of the virus particle through a two-cycle differential centrifugation strongly indicates that it is connected with the virus particle itself. It was shown

that the capacities of the virus particle (*a*) to infect, (*b*) to destroy the inhibitory activity and (*c*) to combine with the inhibitor or to haemagglutinate may be successively destroyed by suitable treatment.

Trypsin digestion of the inhibitor obviously causes an appreciable reduction in inhibitory activity. Contrary to the previously cited observations, it was, however, found impossible to destroy completely the inhibitory activity, whether by increase of enzyme concentration or by prolongation of the incubation. The reaction rate was relatively rapid at the start and then progressively decreased. This coincides with the assumption that the inhibitor substance is enzymatically broken down. Also there is nothing to indicate that the decrease of inhibitory activity is caused only by a block of the reactive groups through a combination of inhibitor and trypsin.

As an explanation for this phenomenon two possibilities immediately suggest themselves :

(*a*) Provided all receptor groups have a similar chemical structure, the trypsin digestion apparently causes a decrease only of their reactivity without complete loss of their capacity to combine with virus. Evidence that there is such a modification of the receptor groups may be found in the observation that the trypsin effect seems to be greater if measured with active virus than with inactive (Tables II and IV), which may indicate more rapid destruction of the inhibitor by the viral enzyme following trypsin treatment.

(*b*) Another possibility is that the inhibitor effect refers to two or more chemically different substances with different resistance against trypsin digestion. In this connection it may be pointed out that the inhibitor preparations used contained several components that could be differentiated by sedimentation analysis. Although previous observations indicate that the inhibitory activity should be associated only with the so-called 200S component, it seems not unlikely that these large particles might contain chemically different receptor groups. Further investigation is obviously needed.

Treatment with periodate in concentrations above M/800 completely destroyed the combining capacity of the inhibitor. Compared with the limited effect of trypsin, this indicates that carbohydrate is more essential than the protein part of the receptor complex.

The observations of Burnet (1948*b*) and Fazekas (1949) that treatment with moderate concentrations of periodate modifies the receptor, rendering it non-digestible by the viral enzyme, have been partially confirmed. Treatment of the inhibitor with periodate within a low, very narrow concentration zone of periodate prolonged the time needed for dissolution of a precipitate of this modified inhibitor and active standard virus. The dissolution reaction was, however, never completely prevented; the reaction time was only prolonged. As the precipitation-dissolution reaction in all other cases seems to reflect the course of the combination-elution reaction between virus and inhibitor, it may be assumed that these modifying doses of periodate, somehow, altered the inhibitor particles, rendering them less sensible to the presumptive enzyme of the standard virus.

Actually, Fazekas (1949) showed only that no significant elution of virus took place in a period of time that exceeded only slightly the time needed for elution in the controls. The above data suggest that a much longer incubation might be needed for elution of virus from modified receptors. If, thus, the viral enzyme action is only retarded by modification of receptors, then the observation

of infection through modified receptors does not prove the dispensability of the viral enzyme action on the receptors for the initiation of influenza virus infection.

It is tempting to speculate about the implications of the fact that the periodate almost completely dissolved the precipitate while the active virus had only a limited effect.

If we consider the special relationships in the virus-inhibitor complex, it is easy to visualize how the small periodate molecules can gain access to the reactive sites of the inhibitor, whereas a similar effect of the large virus particle seems to require dissociation of the virus-inhibitor bonds. Hirst (1949), on the basis of such considerations, arrived at the conclusion that the combination of inactive virus and inhibitor represents an association-dissociation equilibrium. The fact that in the experiments described above only a minor part of the virus was released suggests that certain of the bonds between virus particles and inhibitor might be stronger than others. At any rate a complete dissociability of the inhibitor-inactive virus complex seems unlikely.

SUMMARY.

Observations are reported regarding the modified course of the interaction between purified concentrates of influenza virus and the inhibitor of normal allantoic fluid by suitable treatment of the inhibitor.

Treatment of the inhibitor even with small amounts of active virus gradually destroys both the haemagglutination inhibitory capacity and the capacity to precipitate with high concentrations of active or inactive viruses.

Sodium periodate in rather low concentrations also destroys the inhibitory and precipitating capacities of the inhibitor particles. In a lower, very narrow concentration zone of periodate, however, the precipitate with active standard virus was formed as usual, but its dissolution took a longer time.

Proteolytic enzymes, as crystalline trypsin and ficin, on the other hand, even in relatively high concentrations fail to abolish the capacity of the inhibitor particles to precipitate with active or inactive viruses, the precipitate with active virus redissolving in a typical way when incubated. Correspondingly, trypsin diminishes the haemagglutination inhibitory capacity only to 5–25 per cent of the original.

A stable precipitate of inactive virus and inhibitor could be partly dissolved by treatment with low concentrations of active virus or periodate. Periodate released about 2/3 of the haemagglutinins from a fresh complex, only a small part of the precipitate remaining. Active virus left the precipitate at roughly the same level, only about 1/4 of the haemagglutinins being released.

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EXPERIMENTAL LIVER CIRRHOSIS IN RATS PRODUCED BY PROLONGED SUBCUTANEOUS ADMINISTRATION OF SOLUTIONS OF TANNIC ACID.

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IT has been shown that a cirrhosis, resembling human cirrhosis of the Laennec type, can be produced in experimental animals by abnormal diets (Glynn, Himsworth and Lindan, 1948; Wahi, 1949). On the other hand, Cameron and Karunaratne (1936) and Steinberg and Martin (1946) have shown that the experimental cirrhosis of the liver may, in its reversible stage, end in complete anatomical recovery. These facts gave rise to the hope that appropriate dietetic treatment might be successful in human cirrhosis. It is now well known that the ingestion of a suitable diet and lipotropic substances frequently exerts a favourable effect on human cirrhosis, which can be confirmed histologically. Unfortunately, the same therapy may fail completely in other, occasionally not advanced cases. These facts indicate, in our opinion, that cirrhosis of the liver is not invariably a deficiency disease, but that other factors can play a part in its aetiology.

One of us (B. K.) assumed as early as 1942, independently of English (Cameron, Milton and Allen, 1943) and American authors (Wells, Humphrey and Coll, 1942), on the basis of the changes found in the liver of persons who had suffered burns and been treated with tannic acid, that tannic acid had a hepatotoxic effect. He administered tannic acid solution parenterally to experimental animals and found central acinar necrosis in the livers of those animals which died after 24 to 26 hours. Vascular phenomena simulating the pattern of a serous hepatitis may be observed before the development of the parenchymal lesion (Korpássy, 1949).

The hepatotoxic effect of tannic acid having been proved, the question of what effect the protracted treatment with sublethal doses of tannic acid would have on the liver arose, knowing that acute poisoning leads to central acinar necrosis.

EXPERIMENTAL METHODS.

Twenty white rats aged about 3 months were used. Their average weight was 112 g., varying between 90 g. and 130 g. Male and female animals were used; they were all of the same strain, the origin of which was not known. The animals received a mixed diet of waste food from the hospitals, consisting mainly of milk-bread, potatoes, farinaceous food and sometimes fresh curd. No accurate diet could be maintained, so white rats, untreated or used for other experiments, were kept on the same diet and served as controls. Changes in the liver parenchyma could not be found either in the untreated rats or in those employed in other experiments, whether they died or were killed, though several animals were rather old at the time of the examination. The untreated animals grew and reproduced normally on this diet.

Treatment.

All rats were given 10 mg. of tannic acid on days 1, 3, 5 and 7 of the experiment. On days 9, 10, 15, 17, 19, 29, 51 and 53 the dose given was 20 mg., on days 60, 61, 93, 102, 109, 114 and 116 the dose was 30 mg., on days 65, 69, 84, 118, 123, 126, 132, 137 and 142 the dose was 40 mg., on days 147, 153, 159, 165, 172 and 194 the dose was 50 mg., on days 200 and 208 the dose was 60 mg., and on day 215, 70 mg. were given. The tannic acid was administered subcutaneously as a 1 per cent solution for the first 10 doses, and thereafter as a 2 per cent solution. Four animals died on day 19, 2 on day 29, 2 on day 31, 1 on day 34, 2 on day 39, and 1 on each of days 70, 71, 85, 94, 217 and 232. On each of days 69, 141 and 195 a rat was killed. The average weight fell from 112 g. to 96 g. in 19 days. On day 39 the average weight was 120 g., on day 69 it was 152 g., on day 102 it was 175 g., on day 137 it was 187 g., on day 166 it was 176 g., on day 190 it was 180 g., and on day 210 it was 195 g.

Necrosis occurred at the site of the subcutaneous injections after the 20th day, and ulcers 2 to 3 cm. diameter were left after separation of the necrotic tissue. All the ulcers healed without special treatment, and no inflammation of their margins or in the deep layers was observed. In no case could the death of the animal be attributed to these local processes.

All the animals were dissected as soon as possible. The organs of 18 rats were examined; 2 rats which died on days 19 and 39 had been devoured by the others.

Gross and microscopic changes in the liver.

In order to obtain a clear picture of the changes due to the prolonged treatment the animals were grouped according to the time of their death. The first group contained the animals which died up to the 39th day; the second group contained those which died between days 69 and 94; the animals in the third group survived 100 days.

Group I (9 rats).—The liver was slightly swollen, with a smooth surface, and had the structure of nutmeg liver in miniature. Histological examination showed a varying degree of central breakdown of the lobules. The débris had for the most part been removed and its place taken by red cells, resulting from dilatation or rupture of the central vein and neighbouring sinusoids. In the livers of those animals that died between 20 and 26 hours after the last treatment, karyorrhexis of the central cells could be seen. In the peripheral zone of the lobules the normal radial structure had disappeared. The liver cells were generally enlarged, nevertheless they displayed striking variations in size, and there were numerous cells in which both the cytoplasm and the nucleus were enormously enlarged (Fig. 1). In addition, double nuclei and cell divisions could be seen. The nuclear staining was uneven ; liver cells with pale vesicular nuclei were interspersed with others whose nuclei were hyperchromatic and pyknotic. Not infrequently round acidophil inclusion bodies could be seen in the vesicular nuclei. Large vacuoles appeared in the cytoplasm of some cells, but fat in small droplets could rarely be demonstrated in the sections with Sudan III. In some cases where there had been central haemorrhagic necrosis, accumulations of haemosiderin were present in the parenchymal cells. In other cases the cells contained light yellow granular bile pigment.

There were other changes deserving attention. As early as the 19th day there were in the sections small or medium-sized elongated cells with a bright staining nucleus, and these cells were more numerous in the livers of the animals which died later. These cells were arranged in short or long columns and strands between the remaining cords of liver cells. Sometimes they were situated on one side of the dilated central vein like a crescent or cap and they seemed to invade the liver tissue from here. As a rule, the number of these cells was increased in the periportal tissue also.

The behaviour of the reticulum as shown by Gömöri's impregnation was rather characteristic. Initially, the fibrous meshwork was collapsed in the region of the central acinar necrosis and broken to pieces on the periphery (Fig. 4). Later on, after the 30th day, the primary thickening of the reticulum fibres could be seen, mainly around the central veins, and to a lesser degree around the interlobular vessels (Fig. 5).

Group 2.—In 2 of the 5 rats belonging to this group (they died on days 71 and 85 respectively) the gross changes were well marked. The liver was enlarged

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EXPLANATION OF PLATES.

FIG. 1. Rat T/11. Treated with 180 mg. tannic acid administered in 11 doses. Died on 29th day. Haematoxylin and eosin. × 210.

FIG. 2.—Rat T/6. 18 injections, total 400 mg. tannic acid. Died on 85th day. Haematoxylin and eosin. × 360.

FIG. 3.—Rat T/18. 28 injections, total 750 mg. tannic acid. Killed on 141st day. Haematoxylin and eosin. × 54.

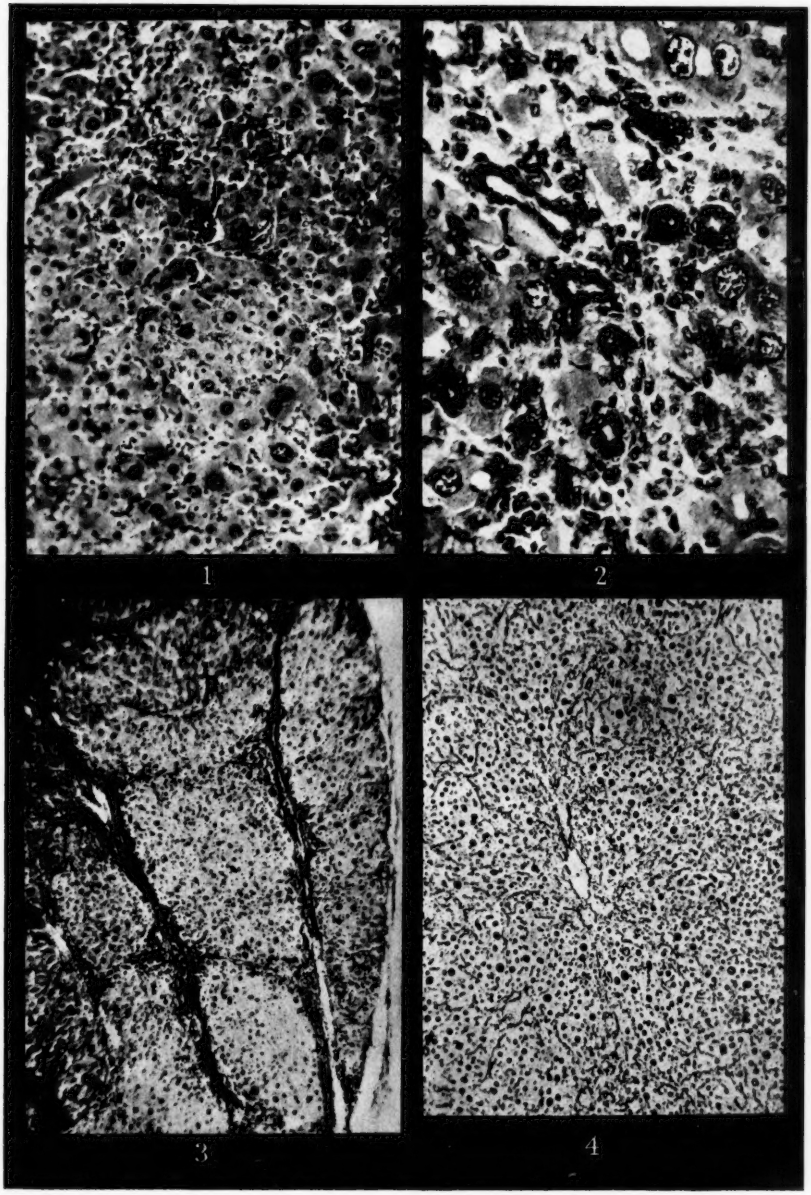
FIG. 4.—Rat T/11. 10 injections, total 160 mg. tannic acid. Died on 19th day. Gömöri's reticulum stain. × 57.

FIG. 5.—Rat T/13. Treated with 180 mg. tannic acid administered in 11 doses. Died on 31st day. Gömöri's reticulum stain. × 57.

FIG. 6.—Rat T/5. 16 injections, total 320 mg. tannic acid. Died on 69th day. Gömöri's reticulum stain. × 57.

FIG. 7.—Rat T/18. 28 injections, total 750 mg. tannic acid. Killed on 141st day. Gömöri's impregnation. × 57.

FIG. 8.—Rat T/19. 38 injections, total 1280 mg. tannic acid. Died on 217th day.



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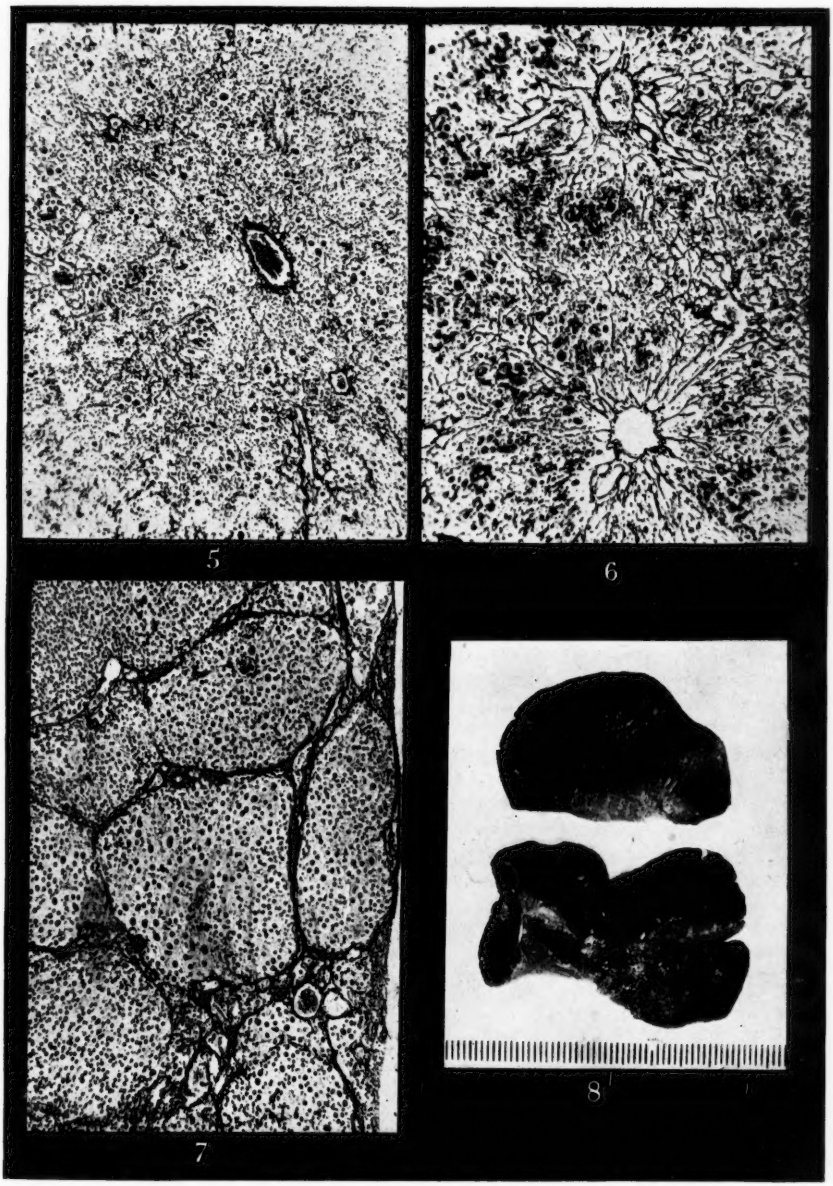

Korpássy and Kovács.

and firm with its surface finely and evenly granular, especially when seen with a lens. The breakdown of the architecture of the liver was conspicuous in all cases and the polymorphism of the liver cells was more marked than in Group 1. The elongated cells mentioned under Group I were present in enormous numbers and not infrequently they formed thick strands extending from the centre of one acinus into another. In other places these cells were oval or flattened and here and there formed wreath-like structures.

The bile ducts were invariably increased in numbers, and groups of 8 or 10 were sometimes found between the lobules or in the interior of the disintegrated lobules. These bile ducts, some of which had no apparent lumen (Fig. 2), were often surrounded by the elongated cells previously mentioned. The reticulum fibres were likewise increased in numbers and thickened. The gradual transformation of the architecture of the liver could be followed well in sections stained by Gömöri's impregnation, a method which demonstrates the liver cells in addition to the reticulum. The thickened and more numerous reticulum fibres, forming strands of varying thickness, delineated and clearly indicated the irregular disintegrated lobules, and here and there surrounded groups of surviving liver cells (Fig. 6).

Sections of the liver of the rat which died on the 85th day, stained by van Gieson's method, showed collagen fibres in some of the thickened strands of connective tissue. Traces of fat and glycogen could also be found.

Group 3 (4 animals).—In 3 animals (treated for 141, 195 and 217 days respectively) it was obvious on external examination of the liver that it was diseased. The surface was finely and evenly granular, the greyish-brown nodules about the size of millet seeds were sharply defined and were separated by dark coloured furrows (Fig. 8). The cut surface showed that the granularity extended uniformly throughout the organ.

The histological picture was very similar to that of human cirrhosis of the Laennec type. The nodules corresponded to groups of liver cells surrounded by bands of connective tissue of varying thickness. These nodules are pseudo-lobules ; the hepatic cells are not arranged in any definite fashion, and there is no central vein or the vein of the lobule is situated at the periphery (Fig. 3). The cells constituting these pseudo-lobules were markedly polymorphic, but the endothelium lining the rather wide sinusoids appeared normal. The pseudo-lobules had a delicate reticulum meshwork, in sharp contrast to the coarser surrounding fibres (Fig. 7). The small elongated cells were increased in number only in the environment of the liver cell groups. In two cases the proliferation of the bile ducts resulted in structures resembling adenomas.

In the liver of the rat which survived all the others, moderate fibrosis was present, but the characteristic distortion of the architecture was absent.

RESULTS.

Histological examination has shown that the protracted *parenteral* administration of tannic acid results in a gradually progressive destruction of the liver parenchyma and transformation of its architecture. In the early stages breakdown and subsequent regeneration of the liver cells are the most striking features ; direct and indirect cell divisions may be seen in the marginal cells of the lobules, while other nuclei are, owing to their inner division, enormously enlarged. Changes

in the reticulum seem to be a good measure of the degree of transformation of the liver architecture. During the first week or two the reticulum fibres are moderately thickened and increased in numbers, particularly around the central veins. By the 70th day the increase and thickening of the reticulum is considerable and the broken down liver parenchyma is interwoven by strands of reticulum of varying thickness which split off pieces of varying size and shape from the lobules. At the same time the proliferation of the bile ducts sets in. This stage corresponds, in our opinion, to an early cirrhosis or precirrhosis.

In rats which survived 100 days of treatment the architecture of the liver was already distorted or completely transformed. The remnants of the parenchyma form round nodules of various sizes surrounded by a connective tissue meshwork containing collagen. The proliferation of the bile ducts simulates adenoma at many places. The changes seen in the liver of the rat killed on day 141 correspond to a diffuse fibrosis indistinguishable from the classical pattern of portal (Laennec) cirrhosis.

We attribute a particular role in this transformation of the liver structure (tannic acid cirrhosis) to the small elongated cells which were observed initially in small, later in gradually increasing, numbers. These cells are, in our opinion, of reticulo-endothelial origin and they seem to play some part other than reticulum formation. Our serial sections seem to indicate that these small immature cells may develop into bile ducts (i.e. epithelial cells of the bile ducts) and also occasionally those small liver cells which could be found in their environment. The presumption may be warranted that this strong hepatotoxic compound, or perhaps the repeated destruction of the parenchyma, has the sequel that the remaining reticulo-endothelium takes on the pluripotential properties of the embryonal mesenchyma.

DISCUSSION.

There are numerous papers in the literature dealing with the experimental production of cirrhosis. Besides the purely dietetic procedures mentioned in the introduction to this paper, there are many organic and inorganic substances—drugs, tar-like materials, proteins and their decomposition products, bacterial toxins, etc.—which produce liver lesions when administered to experimental animals (Moon, 1934). The results of these experiments are not quoted here as the lesions reported are quite unlike the classical human cirrhosis of Laennec type.

Mallory (1925, 1933 ; Mallory and Parker, 1931) was engaged in the problem of the aetiology of cirrhosis of the liver for several decades. Having ruled out ethyl alcohol as exclusively responsible, as other authors such as Friedenwald (1905) had also done, he performed extensive experiments to examine the effects of those substances which might contaminate alcoholic beverages. He examined more than 30 substances, including copper, lead, aluminium, antimony, zinc, arsenic, sulphates, furfurol, turpentine, and creosote. Ultimately, on the basis of his experiments in 1933 with phosphorus, he claimed that Laennec's cirrhosis was due to the contamination of beverages with phosphorus. However, the experimental phosphorus cirrhosis produced by Mallory bears little relation to human cirrhosis of the Laennec type.

There are, in fact, not many chemical substances capable of producing cirrhosis in a strict sense in animals. Beattie and Dickson (1948) hold that manganese

and its salts belong to the small group of substances which produce a hepatic lesion resembling human cirrhosis (Findlay, 1924; Hurst and Hurst, 1928). Many authors have of recent years been concerned with the effect of carbon tetrachloride (Cameron and Karunaratne, 1936; Sundareson, 1942; Ungar, 1945; and others). Ashburn, Endicott, Daft and Lillie (1947), unlike the other authors, contend that the first alteration due to carbon tetrachloride is proliferation of the connective tissue around the central veins. Since the discovery of Kinoshita (1937) attention has been directed to the cirrhogenic and carcinogenic effect of the azo dyes, especially butter yellow. The combination of different agents has proved more effective than the application of the individual substances (Moon, 1934).

Having compared the histological changes resulting from the protracted subcutaneous application of tannic acid, butter yellow, and other substances it appeared to us that the cirrhoses produced by carbon tetrachloride and butter yellow bore the greatest resemblance to tannic acid cirrhosis. The cirrhoses due to tannic acid and carbon tetrachloride may have a similar histogenesis, for in both the transformation of the architecture of the liver seems to start from the centre of the lobules. Nevertheless, wherever the process which results in diffuse fibrosis may begin, the final pattern cannot be distinguished from that of classical portal cirrhosis, as has recently been stressed with regard to dietetic cirrhosis (Glynn, Himsworth and Lindan, 1948).

Finally, the question arises whether the various substances which can produce cirrhosis in experimental animals play any part in the genesis of human cirrhosis. The majority probably play no part, but no definite opinion as to the aetiological role of tannic acid can be formed for the time being. Further investigations are being carried out in order to answer, amongst others, the following questions: (1) Have the skin necroses and ulcerations, which inevitably occur with the subcutaneous applications of tannic acid, any influence on the development of the cirrhosis? (2) Is the effect of oral administration identical with that of injection? In any case, we feel that the recognition of the fact that tannic acid can produce cirrhosis in experimental animals opens up a further line of investigation into this disputed problem.

SUMMARY.

1. The authors have produced diffuse nodular fibrosis of the liver in rats by the prolonged subcutaneous administration of tannic acid at various intervals.

2. As a result of the repeated administration of tannic acid, a gradual breakdown of the parenchyma and a progressive transformation of the liver architecture takes place. The first sign of this process is an increase of the reticulum around the central veins, corresponding with the central acinar destruction of the parenchyma.

3. By the 70th day of treatment fibre strands connecting adjacent lobules have been developed, and at the same time the regenerative proliferation of the bile ducts becomes conspicuous (precirrhosis).

4. The picture observed on the 141st day was indistinguishable from classical portal cirrhosis (Laennec type).

5. All rats dying between days 69 and 94 of the treatment developed changes corresponding to precirrhosis, whereas in 3 of the animals surviving 100 days a diffuse nodular cirrhosis was apparent on gross examination.

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THE KAOLIN-ADSORPTION METHOD FOR THE QUANTITATIVE ASSAY OF URINARY GONADOTROPHINS.

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THE concentration of gonadotrophic substances in normal male and female urine is so low, that for their assay concentrated extracts must be prepared, freed from toxic substances and other hormones, especially oestrogens and androgens. It has been found that the gonadotrophic substances of menopausal or castrate urine, and probably of normal urine as well, are more susceptible to any drastic extraction process than those of pregnancy urine. For example, 60 per cent acetone containing 4 per cent ammonia destroys the menopausal hormone but not the pregnancy hormone (Evans and Simpson, 1934). Therefore, mild methods of extraction must be used.

Crude extracts may be prepared by the original alcohol-precipitation method of Zondek (1930) as modified by Leonard and Smith (1934), Frank, Salmon and Friedman (1934), Heller and Heller (1939), Heller and Chandler (1942), Frank and Berman (1939) and Frank (1939), Varney and Koch (1942) and many others, by the tannic precipitation method elaborated by Levin and Tyndale (1936, 1937) or by the method involving adsorption on benzoic acid originated by

Katzman and Doisy (1932, 1934). The extracts prepared by these methods cannot be injected into an animal in the doses equivalent to large amounts of urine, as is necessary with normal male or female urine, unless their toxicity is reduced. Some detoxicating procedures, such as dialysis, decrease not only the toxicity of the extract but also the activity. The ultrafiltration method (Jungck, Maddock and Heller, 1947) seems to be even less efficient quantitatively than the alcohol-precipitation-dialysis procedure.

Scott (1940), for purposes of biological pregnancy diagnosis, adsorbed the chorionic gonadotrophin in diluted urine on kaolin. Water soluble toxic substances, such as excess of inorganic salts (Main, 1939), remained in the discarded supernatant. The hormone was extracted from the kaolin precipitate with alkali and after acidification injected into rabbits, mice, or toads. This method has been adopted to the assay of gonadotrophins in normal male and female urine. The proteins in the alkaline extract were freed from steroids by precipitation with acetone after acidification. The final, relatively non-toxic, crude concentrate of any urine can be quantitatively bioassayed, the yields being apparently sufficient to make further purification, isolation and standardization more practicable.

METHODS.

Extraction of urinary gonadotrophins.

There are three steps in the method used : adsorption of the hormones from diluted urine with kaolin at pH 4·0, extraction of the hormones from the kaolin by N/10 NaOH, and precipitation of the hormones by cold acetone, the precipitate being washed with ether.

In preliminary work to identify the gonadotrophic effects, 1 litre of urine was taken from a fresh 24-hour (or on some occasions 48-hour) specimen, diluted with an equal volume of distilled water and acidified with 20 per cent HCl to pH 4·0 (glass electrode). Well-shaken 20 per cent aqueous suspension of kaolin B.D.H. washed with acids (2·5 g. of kaolin per 100 c.c. of native urine) was added to the urine, the mixture being shaken vigorously on the shaking-machine for 1 hour and then allowed to stand for a few hours. When the kaolin had settled down, the clear supernatant was syphoned off and the kaolin suspension transferred to round-bottomed centrifuge tubes and centrifuged at 2500–3000 r.p.m. for 10 min. The supernatant fluid was poured off and the kaolin-precipitate carefully ground up with N/10 NaOH (6 c.c. per 100 c.c. of original urine) until the suspension became homogeneous and free from all lumps. The alkaline kaolin-suspension was then centrifuged for 10 min. at 3000 r.p.m. and the supernatant fluid (about 70 c.c. in volume), containing gonadotrophins, was collected and acidified to approximately pH 5·5–6·0 with 20 per cent HCl, drop by drop. Five volumes (about 350 c.c.) of cold acetone were added to the extract, shaken vigorously and centrifuged for 10 min. at 3000 r.p.m., the acetone being decanted and the precipitate washed with ether (pro narcosi) and dried for at least 1 hour, in a vacuum desiccator in the presence of P_2O_5 and H_2SO_4. To remove the last traces of the solvents the precipitate was dried for 24 to 48 hours in a vacuum desiccator yielding "crude gonadotrophins," a brown-coloured amorphous solid weighing up to 400 mg. per litre of urine. For quantitative assays the total 24 hour urinary specimen was used and extracted likewise.

For the injection the fresh precipitate or "crude gonadotrophins" were suspended or dissolved in 5 c.c. or more 0·9 per cent saline, 1 c.c. of the extract (adjusted if necessary to approximately pH 6·0–6·5) being equivalent to 200 c.c. or less of urine. Usually both the preparation and the first injection of the fresh extract were accomplished on the same or on the next day.

Identification of gonadotrophins.

Only normal immature 21-day old albino rats from Wistar stock, fed on an Aberdeen standard diet, were used. For the purposes of the experiments, no attention was paid to the litters. The extracts were tested for their primary and secondary effects on the weight of the ovaries, testes, uterus, prostate and seminal vesicles. In each animal the organs were examined macroscopically, and usually microscopically and vaginal smears were carried out in all female rats. The extracts were injected subcutaneously into groups of five rats in three equal doses spread over three days, unless the contrary is stated. The vaginal smears were examined five times within 72 to 120 hours after the first injection. On some occasions at this time female rats were vitally stained with 2 c.c. 2 per cent trypan blue injected subcutaneously.

Male rats were killed 72 hours and female 120 hours after the first injection, the reproductive organs being fixed in Bouin's fluid overnight and then dissected, drained on filter paper and weighed on a torsion balance. Before weighing, the ovaries and testes were decapsulated and the uteri were cut off near the cervix and the oviduct, and the intra-uterine fluid if present squeezed out. The lobes of the prostate were separated from the urethra and the whole prostate was weighed, the seminal vesicles being dissected and weighed separately. At necropsy the weight of the control and treated animals was from 35 to 50 g. For the average weight of dissected organs see Tables I and II.

The morphological effects of the concentrates of urine were compared by complete sections of both the experimental and control gonads. The other organs, fixed already in Bouin's fluid were also cut in serial paraffin sections. The haemalum-eosin method and azocarmine-aniline blue and orange-G method were used for general microscopic examination. The contrast staining for study the ovaries of the rats vitally stained with trypan blue was carried out with an aqueous solution of safranin.

Quantitative bioassay.

Mouse uterine technique (Levin and Tyndale, 1936, 1937) was adopted. Normal immature 21-day old female mice weighing from 7 to 10 g. were used. The extracts were injected subcutaneously once daily up to 0·6 c.c. at 24 hour intervals on three consecutive days. The animals were killed 72 hours after the first injection, the uterus being removed, dissected, freed from intra-uterine fluid by pressure between pieces of filter paper, and while fresh weighed on a torsion balance. At necropsy the ovaries were examined and on some occasions also dissected and weighed. Two to five mice were used on each dosage level. The weight of killed animals was from 8 to 12 g. For the average weight of dissected organs see Tables III and IV.

The mouse (uterus) unit was defined as the amount of activity to produce 72 hours after the first injection a 100 to 150 per cent increase over the controls in the average uterine weight of those treated with extract.

Recovery experiments.

These experiments tested the recovery from 0·9 per cent saline and fresh urine of a normal girl of added "crude gonadotrophins" from the urine of postmenopausal women. The final dried postmenopausal precipitate weighing 382·42 mg. was dissolved in 18 c.c. of distilled water and divided into three portions each of 6 c.c. in volume. One portion served as a standard preparation the second was dissolved in 100 c.c. of 0·9 per cent saline and the third in 100 c.c of urine, in which the actual gonadotrophin content was determined. Both the saline and urinary specimens with the postmenopausal gonadotrophins added were put through the kaolin adsorption procedure and then the reprecipitated gonadotrophins dissolved up to the original volume of 6 c.c. of 0·9 per cent saline and quantitatively assayed in comparison with the original gonadotrophin content of both the postmenopausal and normal girl urine.

RESULTS.

Effect of urinary gonadotrophins on immature rats.

The average results of these experiments are given in Tables I and II. The microscopic changes are shown in Fig. 1 to 11. The figures representing the average of the number of rats and their responses on each dose level shows the reliability of the results obtained with the kaolin-adsorption method as compared with other methods.

TABLE I.—*Response of Female Reproductive Organs in Normal Immature Rats to Concentrates of Urinary Gonadotrophins.*

Subject, Sex, Age. (M) = male. (F) = female.	No. rats.	Treatment. Total dose c.c.	Treatment. Equivalent of urine c.c.	Treatment. Vaginal smears* +,±,−	Necropsy: Mean weight of Body g.	Necropsy: Mean weight of Uterus mg.	Necropsy: Mean weight of Ovaries mg.	Increase over controls. Uterus per cent.	Increase over controls. Ovaries per cent.
	20	—	—	—	*48·7*	*18·8*	*10·6*	—	—
W. (F) 60	5	0·5	50	±	48·0	57·6	20·0	205·3	88·6
	5	1·5	150	+	49·4	73·0	35·0	288·2	230·1
	5	2·0	200	+	36·2	78·6	42·2	318·0	279·2
S. (F). 72	5	1·65(1)	200	+	48·0	84·0	44·6	346·8	320·7
G. (F) 56	5	2·0	200	+	47·8	80·0	40·6	325·5	283·0
M. (F) 73	1	1·0(2)	200	+	46·0	80·0	40·0	325·5	277·3
	1	1·5(2)	300	+	40·0	85·0	48·0	352·1	352·8
	5	2·0(2)	400	+	36·0	83·3	50·0	343·0	371·7
L. (M) 17	5	0·5	50	−	47·4	18·6	10·6	—	—
	5	1·0	100	−	46·2	22·4	11·0	19·1	3·7
	4	1·5	150	±	40·2	26·7	12·8	42·0	20·7
	5	2·0	200	+	38·6	47·8	17·6	154·2	66·0
A. (F) 22(3)	5	2·0	200	±	45·8	34·8	23·0	85·1	116·9
B. (F) 20(3)	5	2·0	200	±	45·8	28·0	19·2	48·9	81·1
R. (F) 16(3)	5	2·0	200	±	45·4	42·8	22·4	127·4	111·3

(1) 24-hour output = 825 c.c.

(2) Precipitate diluted in 5 c.c. 0·9 per cent saline. Five injections within 3 days. Single results for 1·0 and 1·5 c.c.

(3) Nullipara with regular menstrual cycle. No samples collected on the days 12 and 13 and 20 and 21 after the beginning of menses in order to avoid any interference of increased gonadotrophin content. (d'Amour, Funk and Liverman, 1939; Levin, 1941).

* Cornified cells (only) in none (−), in some (±), in all (+) treated rats.

TABLE II.—*Response of Male Reproductive Organs in Normal Immature Rats to Concentrates of Urinary Gonadotrophins.*

Subject, Sex, Age. (M) = male. (F) = female.	No. rats.	Treatment. Total dose c.c.	Treatment. Equivalent of urine c.c.	Necropsy: Mean weight of Body g.	Testes mg.	Prostate mg.	Seminal vesicles mg.	Increase over controls. Testes per cent.	Prostate per cent.	Seminal vesicles per cent.
	10	—	—	*44·8*	*156·0*	*29·8*	*7·2*	—	—	—
R. (F) 8(1)	5	0·5	50	37·8	160·8	33·8	9·0	3·0	13·4	25·0
	5	1·0	100	43·8	216·6	51·8	10·4	39·0	73·8	44·4
	5	2·0	200	47·0	310·4	85·4	25·0	99·0	186·5	233·3
T. (F) 70	4	2·0	200	42·0	306·5	56·5	8·5	96·4	89·5	18·0
L. (M) 17	5	2·0	200	36·0	240·0	51·6	12·6	53·8	73·1	75·0

(1) Patient with periodical agranulocytosis.

Increasing doses give increasing effects on immature gonads as well as on the uterus, prostate and seminal vesicles. Ovarian weight followed uterine weight with every extract of both the postmenopausal and normal male or female urine. But, while the uterine weight appears first to rise to a peak and then come to a stop at some level which may or may not represent an optimal value, the ovarian weight rose progressively. These responses were similar to those due to injection of pituitary extract but not to those due to injection of pregnancy urinary extract. However, it will be seen that this interpretation might be incorrect if compared with morphological results.

The microscopic examination of reproductive organs and of vaginal smears of the animals injected with large doses of extract showed a usually increased growth and activity of those organs. With the equivalent of 200 c.c. of urine the enlargement of the ovaries was chiefly due to an increase in the number of developing follicles (Fig. 1 and 2). An ovarian response was observed within 120 hours by which time an enormous number of mature uniform follicles developed, but only to degenerate (Fig. 3). Many follicles reached almost simultaneously the Graafian preparatory stage to ovulation, but the actual rupture did not occur. All large follicles showed advanced degenerative changes involving the destruction of the ovum by pyknosis and chromatolysis. Some

EXPLANATION OF PLATES.

FIG. 1.—The ovary of immature rat injected with concentrate equivalent to 200 c.c. of urine of a girl with periodical agranulocytosis (× 20).

FIG. 2.—The ovary of immature rat injected with concentrate equivalent to 200 c.c. of postmenopausal urine (× 20).

FIG. 3.—High power view (× 300) of section shown in Fig. 1.

FIG. 4.—Ovarian follicle of immature rat injected with concentrate equivalent to 200 c.c. of postmenopausal urine, showing macrophages containing trypan blue (× 265).

FIG. 5.—The ovary of immature rat injected with concentrate equivalent to 400 c.c. of postmenopausal urine (× 20).

FIG. 6.—High-power view (× 215) of section shown in Fig. 5.

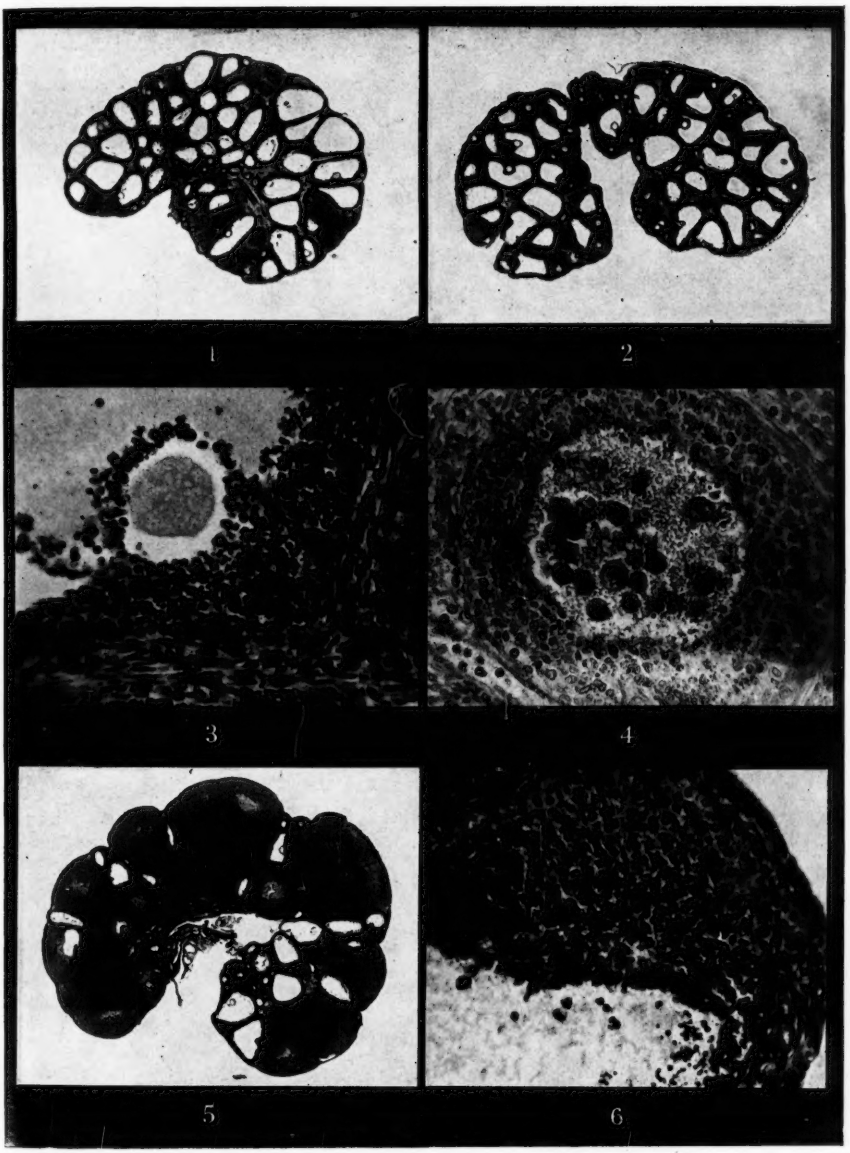
FIG. 7.—Seminiferous tubules of immature rat injected with concentrate equivalent to 200 c.c. of normal male urine (× 230).

FIG. 8.—Seminiferous tubule of immature rat injected with concentrate equivalent to 200 c.c. of postmenopausal urine (× 365).

FIG. 9.—Cervical epithelium of immature rat injected with concentrate equivalent to 400 c.c. of postmenopausal urine (× 265).

FIG. 10.—Cornual epithelium of the same rat (Fig. 9) (× 365).

FIG. 11.—Ventral lobe of prostate of immature rat injected with concentrate equivalent to 200 c.c. of postmenopausal urine (× 320).



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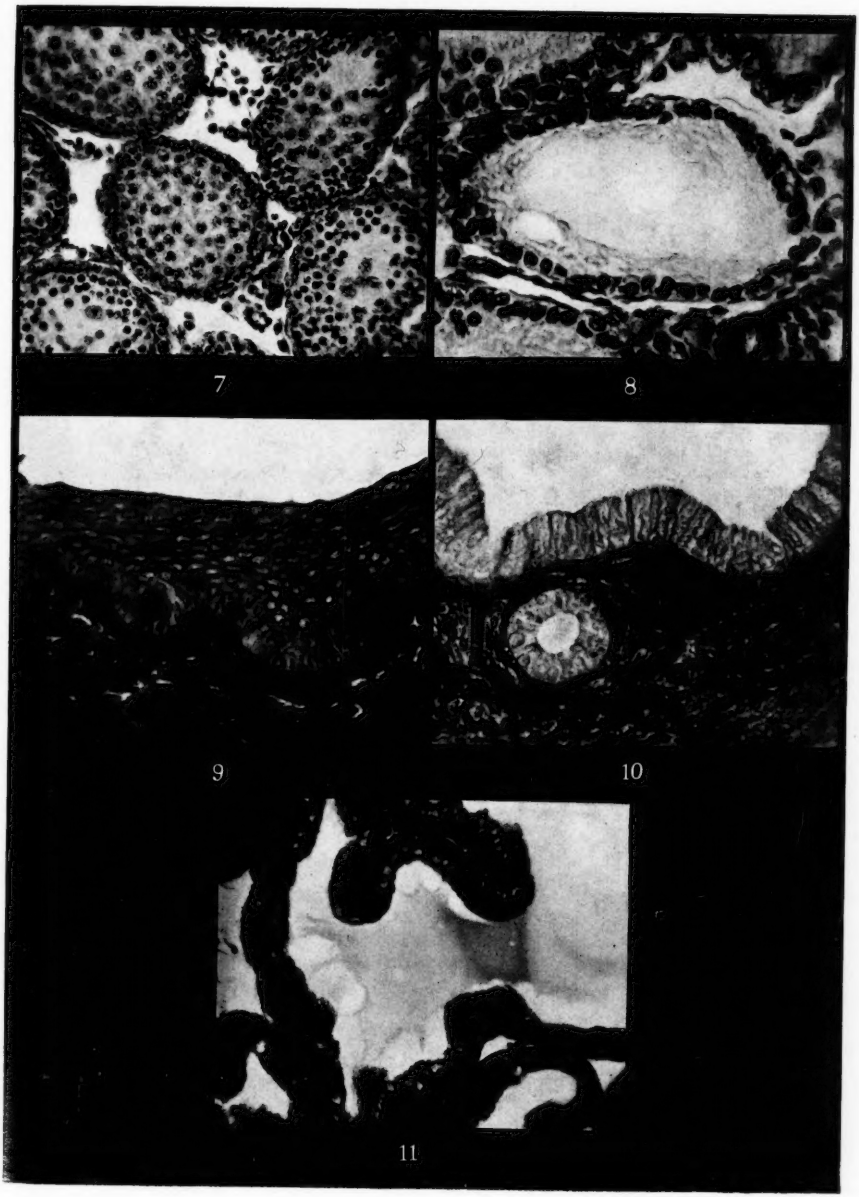

7 8

9 10

11

ova had become detached from the cumulus and lay free in the antrum, in some a polar spindle being present. Cytolysis of the granulosa was observed, the fragments being dispersed into the liquor of the follicles which occasionally were almost deprived of granulosa by this process. Some follicles contained macrophages packed with fragments of nuclear debris and with trypan blue if the animal was vitally stained (Fig. 4). No corpora lutea were found. Only a few primary follicles containing 2 to 7 layers of granulosa cells remained unchanged beneath the tunica albuginea. It was found difficult to differentiate the interstitial cells (gland) as such. With the equivalent of 300 to 400 c.c. of urine a definite thecal luteinization in various phases was observed. The theca interna thickened, the lumina of some follicles being rapidly vascularizated and obliterated (Fig. 5 and 6).

The enlargement of testes was chiefly due to the development of seminiferous tubules with active spermatogenesis, but again with large doses degeneration occurs within 72 hours. Some tubules showed perfectly normal structure. There was a layer of spermatogonial cells followed by a layer of spermatogonia in various stages of meiosis, followed again by spermatocytes showing first stages of spermteleosis (Fig. 7). Spermatogenesis was never seen to end in production of fully differentiated sperms. On the other hand, some tubules showed a completely different picture. Near to the basilar membrane there were Sertoli's cells in usual number and between them spermatogonia, all having nuclei in resting state none of these being of the next stages of spermatogenesis (Fig. 8). In the lumina of those tubules there was seen an eosinophylic fibrinous irregular framework which resembled the syncythial structure of Sertoli's cells. No significant changes were observed in the interstitial cells of Leydig.

The uterus, prostate and seminal vesicles were much enlarged, the uterus occasionally distended with fluid achieving sometimes a 10- to 12-fold size. The most interesting feature observed was a stimulated growth of the cervical epithelium (Fig. 9) and a marked enlargement of cornual glands (Fig. 10). The prostate and seminal vesicles showed an active secretory state close to sexual maturity as judged by distension of the lumina and the light areas (More, Price and Gallagher, 1930) typical of the normal secretory cells of Bouin-fixed ventral prostate (Fig. 11) and the tall columnar epithelium and heightened folds of the seminal vesicles. With the technique used, the typical " secretion granules " of the active cells of seminal vesicles (Moore, Hughes and Gallagher, 1930) were almost undistinguishable.

The release of oestrogen was in addition indicated by the vaginal changes and smears. The immature vagina opened regularly 4 or 5 days after the first injection of large doses with a pro-oestrus smear which changed to a cornified type.

The morphological changes observed indicate that both follicle-stimulating and luteinizing effects were present in those animals and that with excess of urinary concentrates at the same time both follicle stimulation and follicular atresia in various phases were produced, and in the testes the spermatogenesis suppressed.

Quantitative bioassay.

The results confirm that the mouse uterus is much more sensitive to both postmenopausal and normal (male or female) urinary gonadotrophins than the

TABLE III.—*Quantitative Assay of Normal Urinary Gonadotrophins in the Immature Mouse.*

Subject, Sex, Age. (M) = male. (F) = female. 24-hour output c.c.	No. mice.	Treatment. Total dose c.c.	Treatment. Equivalent of urine c.c.	Necropsy: Mean weight of Body g.	Necropsy: Mean weight of Ovaries mg.	Necropsy: Mean weight of Uterus mg.	Increase over controls. Ovaries per cent.	Increase over controls. Uterus per cent.	Mouse uterus unit per 24-hour output. M.U.U.
	10	—	—	*10·6*	*4·2*	*5·8*	—	—	
C. (F) 65 (950)	2	0·045	1·8	9·6	4·5	6·0	7·1	3·4	
	5	0·060	3·2	9·5	5·0	11·6	19·0	100·0	300
	5	0·075	3·9	11·1	5·2	14·0	23·8	141·3	
	4	0·090	4·7	10·0	5·4	28·7	28·5	394·8	
	3	0·120	6·3	11·0	6·0	35·0	42·8	503·4	
T. (F) 70 (910)	5	0·150	7·6	9·3	5·9	30·8	40·4	431·0	
	5	0·225	11·4	9·4	9·4	54·0	123·8	831·0	
	5	0·300	15·2	9·4	9·8	58·8	133·3	913·7	
	5	0·450	22·7	9·7	10·6	61·8	152·3	965·5	
	5	0·600	30·3	9·6	12·0	62·4	185·7	975·8	
	5	0·900	45·5	10·0	13·8	63·0	228·5	986·2	
L. (M) 17 (1100)	3	0·3	18·3	11·0	—	15·0	—	158·6	60
	4	0·6	36·7	11·2	—	24·5	—	324·1	
	4	0·9	55·0	11·0	—	27·2	—	368·9	
	2	1·2	73·3	11·0	—	37·0	—	537·9	
	2	1·8	110·0	11·5	—	43·5	—	650·0	
Y. (F)(1) 16 (940)	4	0·6	31·3	9·0	—	8·2	—	41·3	
	4	0·9	47·0	9·2	—	8·5	—	46·5	
	4	1·2	62·6	9·2	—	13·2	—	129·3	15
	4	1·8	94·0	10·0	—	19·2	—	231·0	

(1) Nullipara with regular menstrual cycle. The sample was collected on the 8th day after the beginning of menses.

TABLE IV.—*Recovery of Postmenopausal Gonadotrophins Added to Saline and Normal Female Urine.*

(Assay in immature mice.)

Subject, Age. 24-hour output c.c.	No. mice.	Treatment. Total dose c.c.	Treatment. Equivalent of urine c.c.	Necropsy: Mean weight of Body g.	Necropsy: Mean weight of Uterus mg.	Increase over controls. Uterus per cent.	Mouse uterus unit per 24-hour output. M.U.U.
	4	—	—	*9·1*	*5·8*	—	
(*a*) C. 65 (1100)	3	0·060	3·7	10·0	14·5	150·0	300
	3	0·075	4·6	11·0	17·0	193·1	
	3	0·090	5·5	8·5	21·0	262·2	
(*b*) Y. 16(1) (680)	3	0·6	57·9	10·0	19·5	236·2	>10
	3	0·75	67·6	10·5	33·0	468·9	
	3	0·9	86·9	10·0	51·0	779·3	
Recovery of gonadotrophins (*a*) from saline	3	0·075	4·6	10·3	14·3	146·5	240
	3	0·090	5·5	10·3	16·3	181·0	
	2	0·120	7·3	10·2	20·5	253·4	
	2	0·180	11·0	10·5	24·5	339·6	
	2	0·3	18·3	10·0	52·5	805·1	
Recovery of gonadotrophins (*a*) from urine (*b*)	3	0·60	3·7	12·0	13·6	134·4	300
	3	0·075	4·6	10·3	15·5	167·2	
	2	0·090	5·5	10·0	19·5	236·2	
	2	0·120	7·3	12·0	24·7	343·1	
	2	0·180	11·0	11·0	57·5	891·3	

(1) Sample collected on the 11th day after the beginning of menses.

rat's uterus and rat's ovary (Table III). On the other hand, increasing doses give increasing effects on the uterus and ovaries exactly as was observed in immature rats. The increase in ovarian weight followed the increase in uterine weight. When the uterine weight reached a plateau level, the ovarian weight still rose progressively. It was found that to increase the weight of uterus by 100 to 150 per cent over that of the untreated controls the equivalent to 3·2 to 3·9 c.c. of urine was necessary for the postmenopausal urine, but about 5 times more for male urine (18·3 c.c. or less) and about 20 times as much for normal female urine (62·6 c.c. or less). These figures as well as recovery figures (Table IV) show the reliability of the results obtained with the kaolin-adsorption method. It was possible to recover 80 to about 100 per cent of the original activity of postmenopausal gonadotrophins precipitate added to saline and normal urine and then putting the mixture through the kaolin-adsorption procedure.

DISCUSSION.

The original method of Zondek (1930) achieves only a five-fold concentration and therefore is not adequate for the detection of minute quantities of hormones present in the urine of normal men and women (Hoffmann, 1944). Leonard and Smith (1934) elaborated Zondek's method for the qualitative detection of the follicle-stimulating principle of postmenopausal urine. Frank, Salmon and Friedman (1934) introduced the use of acetone instead of alcohol. The dialysis of the final extract introduced by Heller and Heller (1939) and Heller and Chandler (1942) diminished considerably the toxicity of the extract for immature rats but this procedure (dialysis, reconcentration by evaporation, etc.) may lead to an appreciable loss of activity (Levin, 1941). In the preliminary experiments dialysis was carried out in this laboratory but was not satisfactory. Varney and Koch (1942) showed that dialysis causes more loss of activity than extraction or reprecipitation. To lessen the toxicity of the extract, Frank and Berman (1939) and Frank (1939) reintroduced the acid alcohol method, using 400 c.c. of urine for examination. They believe that the tannic acid and benzoic acid methods concentrate mainly or exclusively the follicle-stimulating substance and that the acetone and acid alcohol methods, on the other hand, concentrate both follicle-stimulating and luteinizing substances. Varney and Koch (1942) elaborated a method of extraction of the crude alcohol precipitate with 50 per cent alcohol and reprecipitation from the solution with 95 per cent alcohol. They claim that this modification yielded higher gonadotrophin content and less toxic extracts than others. The tannic acid precipitates (Levin and Tyndale, 1936, 1937) have been found even more toxic (Heller and Heller, 1939; Varney and Koch, 1942). The adsorption on benzoic acid causes a considerable loss of gonadotrophic activity and was later abandoned (Katzman and Doisy, 1937). In general, precipitation with alcohol has proved more satisfactory (Klinefelter, Albright and Griswold, 1943) and gives higher yields (Varney and Koch, 1942), than precipitation with tannic acid or adsorption on benzoic acid.

The kaolin-adsorption method involves no special detoxicating procedure, nor any chemical fractionation of extract. The crude precipitate, freed from acetone and ether, if dissolved in saline was found harmless to the immature rat, even in a single subcutaneous dose of 1 c.c. (equivalent to 200 c.c. of urine).

The yields were as high or higher than those obtained with other methods. The recovery experiments proved the reliability of the results obtained with method used. It is unlikely that the extract does contain enough oestrogens or androgens to produce effects directly or indirectly.

The duality of pituitary gonadotrophins as originally suggested by Fevold, Hisaw and Leonard (1931) and supported by Chow, Greep and van Dyke (1939) has been fairly well established by the isolation of pure luteinizing (interstitial cell-stimulating) protein from sheep (Li, Simpson and Evans, 1940*a*, *b*, 1941, 1942), and pig (Shedlovsky, Rother, Greep, van Dyke and Chow, 1940) pituitary, as well as by purification of the follicle-stimulating (gametogenic) hormone from pig pituitary (Greep, van Dyke and Chow, 1940). On the other hand, there is no such evidence for the presence of both gonadotrophins in the normal male or female urinary extracts.

The identification of gonadotrophins of the crude urinary concentrates in these experiments could be only approximate. The results of all tests were in general agreement and there can be little doubt that gonadotrophins were the main active factors. There is no easy way of estimating two gonadotrophins separately if they are both present in the same extract. Although the two hormones have not been separated it seems probable that postmenopausal and normal urinary concentrates contain both the follicle-stimulating and luteinizing substances. The morphological changes observed in various organs in these experiments on intact immature animals provide objective and direct evidence for both follicle and seminiferous tubules stimulation and luteinizing effects but it is obvious that those results obtained on intact immature animals are complicated by the release of both gonadotrophins by the animals' own pituitary (Noble, Rowlands, Warwick and Williams, 1939).

It was shown that the postmenopausal and normal male or female urinary extracts caused a marked growth of large uniform follicles, but none of those at the Graafian stage had ovulated and no corpora lutea could be seen after five days of injection. These results are in agreement with those of Leonard and Smith (1934). The large doses of these extracts (equivalent to 300 to 400 c.c. of urine or more), on the other hand, stimulated not only a great follicular growth but also an extensive thecal luteinization accompanied with cytolysis of the granulosa and ova, the changes being various phases in advanced follicular atresia. These morphological changes are similar to those observed in intact immature animals treated with the extracts of human pregnancy urine and pregnant mare serum, except that there is no such follicular growth in animals treated with pregnancy urine. By analogy, these urinary extracts injected into the intact immature male rats may produce advanced spermatogenesis but again never to a stage of fully differentiated sperms.

The animal tests may be divided according to whether the effect on the intact immature or hypophysectomized animal is primary or secondary. There are no available standards for expressing the results of the bioassay quantitatively in international units. The results are stated in various animal units such as rat units or mouse units arbitrarily defined in terms of the amount necessary to produce a defined response under defined conditions. It is convenient to speak of the bioassay of "follicle-stimulating" and "luteinizing" hormones which in varying amounts are probably present in each urinary extract, but first it should be proved that human urine contains more than one gonadotrophin

and made clear whether the follicle-stimulating hormone produces in female rats the primary gametogenic effect only as it does in male rats or both the primary and secondary (*via* oestrogen release) effects. The reason for choosing the mouse uterine technique (Levin and Tyndale, 1936, 1937) for the "total" gonadotrophins assay was the much greater sensitivity of the mouse uterus to the active substances and the greater accuracy and uniformity of the response to increasing doses of the extracts (Evans, Hines, Varney and Koch, 1940; Varney and Koch, 1942). The uterine response was produced by smaller doses of extract than those necessary to produce follicular enlargement and an increase in ovarian weight. It was found that the equivalent to 3·2 to 3·9 c.c. of post-menopausal urine was sufficient to increase uterine weight by 100 to 150 per cent over that of the untreated controls, while to increase the ovarian weight by 100 per cent the equivalent to 11·4 c.c. of urine was necessary. Both uterine and ovarian weight can be easily assayed with the same extract. Finally, reducing the assay to 72 hours after the first injection seems to be an important factor in minimizing toxic effects.

SUMMARY.

The kaolin-adsorption method as modified for concentrating normal urinary gonadotrophins was found an efficient, simple and quick procedure, the relatively non-toxic concentrates being suitable for further purification, isolation and standardization of the hormones. The figures for various quantitative tests seem to prove the reliability of the results obtained with the method used.

I wish to thank Professor J. H. Gaddum and Organon Laboratories Ltd., for providing facilities for this work, and also Dr. J. A. Loraine, for his helpful co-operation, and Miss M. A. Mackay for her valuable technical assistance.

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THE TISSUE RESPONSE TO HEAT-KILLED STREPTOCOCCI IN THE SKIN OF NORMAL SUBJECTS, AND IN PERSONS WITH RHEUMATIC FEVER, RHEUMATOID ARTHRITIS, SUBACUTE BACTERIAL ENDOCARDITIS AND ERYTHEMA NODOSUM.

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NUMEROUS workers have in the past injected streptococcal extracts or filtrates into the skin of patients suffering from infections with haemolytic streptococci, from rheumatic fever, or from wholly unrelated conditions. Swift, Wilson and Todd (1929) used crude filtrates from 10-day cultures of indifferent or α-haemolytic streptococci; Birkhaug (1929) used culture filtrates, autolysates, and whole cocci from β-haemolytic, α-haemolytic and indifferent strains of streptococci; Gibson and Thomson (1935) used an extract of ground-up haemolytic streptococci; Lyttle, Seegal and Jost (1935) used a streptococcal nucleoprotein fraction; and Taran, Jablon and Weyr (1944, 1945) used " purified M proteins " from 40 different types of Group A streptococci. These workers all studied the skin reactions produced, as evidenced by appearance of odema and erythema. From their results it appears that a considerable proportion of normal persons, and a higher proportion of patients with rheumatic fever or with non-rheumatic haemolytic streptococcal infections, will give skin reactions to some or all of the above streptococcal products. The intensity of reaction is greater during active disease than during convalescence, and both in control and rheumatic or streptococcal groups the frequency and intensity of positive reactions increases with age. Furthermore, although there are undoubted variations in the intensity of reaction produced by extracts from different strains

of streptococcus, there appears to be a wide overlap between different strains, nearly all being capable of giving reactions in some persons.

In none of the above studies is there any mention of histological changes at the sites of injection. It seemed to us to be of interest to study the early reactions and, more particularly, the later histological changes in human subjects injected intracutaneously with whole streptococci of different antigenic constitutions which had been killed by gentle heating. Owing to the liability to cause sensitization by their very injection into the skin, it was necessary to inject the different strains which were to be tested all on the same occasion. It was not practicable, without losing the co-operation of our experimental subjects, to inject more than seven stock strains. These were all derived originally from human sources, and were selected so as to represent Lancefield groups A, B, D and three different Griffiths types, with and without M and T antigens. A strain of *Str. viridans* was also included. The organisms were grown in a medium containing no proteins other than human, in order that complications due to foreign serum or Forssman type antigens should be avoided.

In a few instances patients were tested with strains of non-haemolytic and α-haemolytic streptococci derived from blood cultures of cases of subacute bacterial endocarditis. These strains (Ew, Cu, Pr, Cr, Th) were cultured in small amounts under the same conditions as our standard strains. In one instance a patient (Y) with rheumatic fever was tested with a strain of β-haemolytic streptococcus derived from his own throat cultures.

Selection of patients.

The normal subjects were members of the medical staff or students, and were carefully questioned for any history of rheumatic disease or of streptococcal infection within recent years. The remainder were patients in the wards. Those with rheumatic fever, rheumatoid arthritis and post-streptococcal erythema nodosum were tested during the period immediately following acute manifestation of their disease. The patients with subacute bacterial endocarditis were tested before receiving penicillin treatment.

EXPERIMENTAL.

Selection and culture of streptococcal strains.

The following strains were obtained through the kindness of Dr. Dora Colebrook :

No.	Designation.	Haemolysis on horse blood agar.	Group.	Type and antigen.
1	B 133	Non-haemolytic	D	..
3	Chown	Viridans	..	..
6	848	α-haemolytic	B	..
8	SF 130	β-haemolytic	A	I (M)
9	Smithers	Weak β-haemolytic	A	I (M and T)
11	Wheatley	β-haemolytic	A	4/24 (T)
13	R 491	,,	A	14 (T + ? M)

All strains were passaged three times through mice, and were then grown at 37° C. for 16–18 hours in 1 litre of nutrient broth with addition of 2 per cent

glucose, 2 per cent Na glycerophosphate, 0·5 per cent Marmite, and 10 per cent human ascitic fluid. A very heavy growth was obtained. The streptococci were centrifuged down, washed with saline, and suspended as a thick suspension in M/30 phosphate buffer, pH 7·2. The suspensions were heated at 56° C. for 1 to 3 periods of 1 hour, until a heavy inoculum into broth gave no growth on incubation. They were stored as thick suspensions frozen at — 15° C. For inoculation a fresh dilution was made in sterile M/20 phosphate buffer pH 7·2, so as to contain approximately 500×10^6 organisms per ml. The volume injected into the skin was 0·1 ml., i.e. 5×10^7 organisms.

Technique of inoculation into human subjects.

0·1 ml. of each streptococcal suspension, containing approximately 5×10^7 organisms, was injected into the skin of the inner side of the forearm in a standard pattern. The areas of erythema and oedema were measured, and a subjective estimate made of the intensity of oedema, 1, 2 and 3 days after. On the 11th to the 15th day (or occasionally later), when small firm nodules could generally still be felt at the sites of inoculation, areas of skin about 10×7 mm. were excised under local anaesthesia and placed immediately in Zenker's solution.

After fixation they were bisected, embedded in paraffin and examined in serial or step sections stained with haematoxylin and eosin ; some sections were stained with Giemsa and some with Gram.

RESULTS.

Erythema and oedema.

The erythema and oedema were maximal on the 2nd or 3rd day, and had faded away in most cases by the 4th or 5th day. When pustule formation and local necrosis occurred the contents of the pustule were invariably sterile, and the cells were predominantly neutrophil polymorphonuclear leucocytes.

In order to arrive at some quantitative assessment the intensity of oedema was graded subjectively from ± to +++, and a numerical value from 1 to 6 assigned to correspond with the grades.

EXPLANATION OF PLATES.

FIG. 1.—Skin of a normal volunteer. Intracutaneous inoculation of heat-killed streptococci, stock strain No. 9. Excised after 11 days. Perivascular collection of lymphocytes and larger mononuclear cells. × 210.

FIG. 2.—Skin of a patient (M. Wi-) with rheumatoid arthritis. Intracutaneous injection of heat-killed streptococci, stock strain No. 6. Excised after 9 days. Nodule showing conglutinated collagen fibres. × 210.

FIG. 3.—Skin response of a patient (F. Ew-) with subacute bacterial endocarditis to intradermal injection of heat-killed autogenous streptococci. After 16 days. "Focal oedema" —group of swollen and vacuolar cells. × 420.

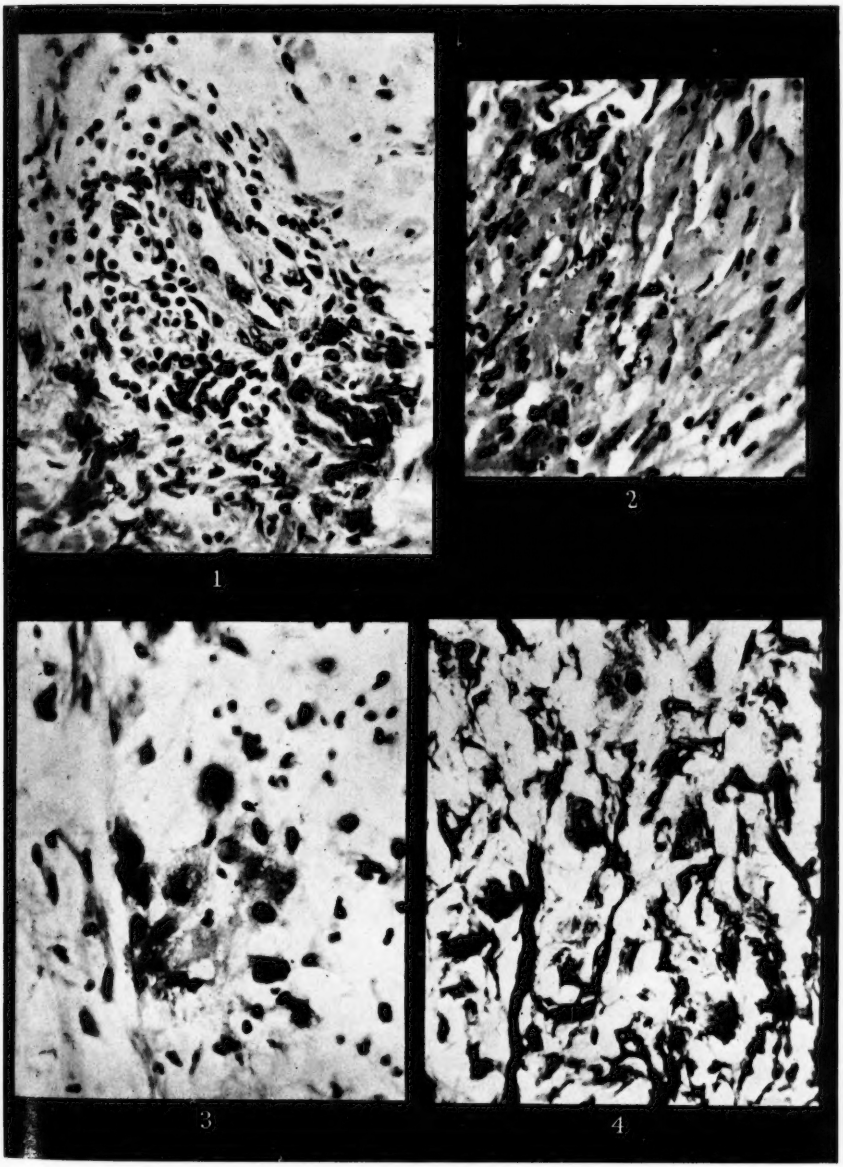
FIG. 4.—The same case. Thickening and conglutination of collagen fibres interrupted by swollen and vacuolar cells. Mallory preparation. × 420.

FIG. 5.—The same case. "Focal oedema" with formation of nodules. × 140.

FIG. 6.—The same case. One of the nodules consisting of conglutinated, swollen and vacuolar cells. × 225.

FIG. 7.—Skin response of a patient (M.B.) with Rheumatic fever ; response to streptococcus 11, on the 18th day. A vessel in the cutis with fibrinoid necrosis in one part of the circumference. × 400.

FIG. 8.—The same. An area showing fibrinoid conglomeration of the collagen. × 400.



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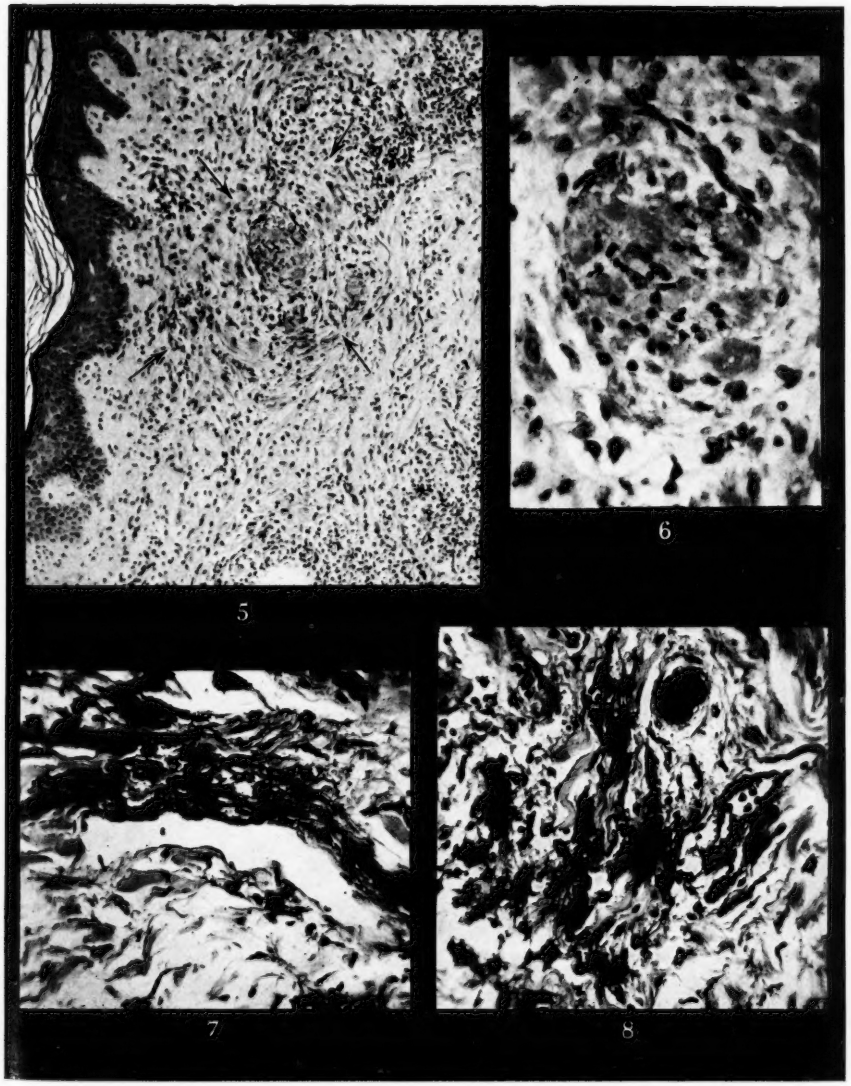

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The information concerning the area and intensity of the reaction to different strains, and the incidence of pustule formation, is summarized diagrammatically in Fig. 9. The mean values only are represented, but there was a considerable

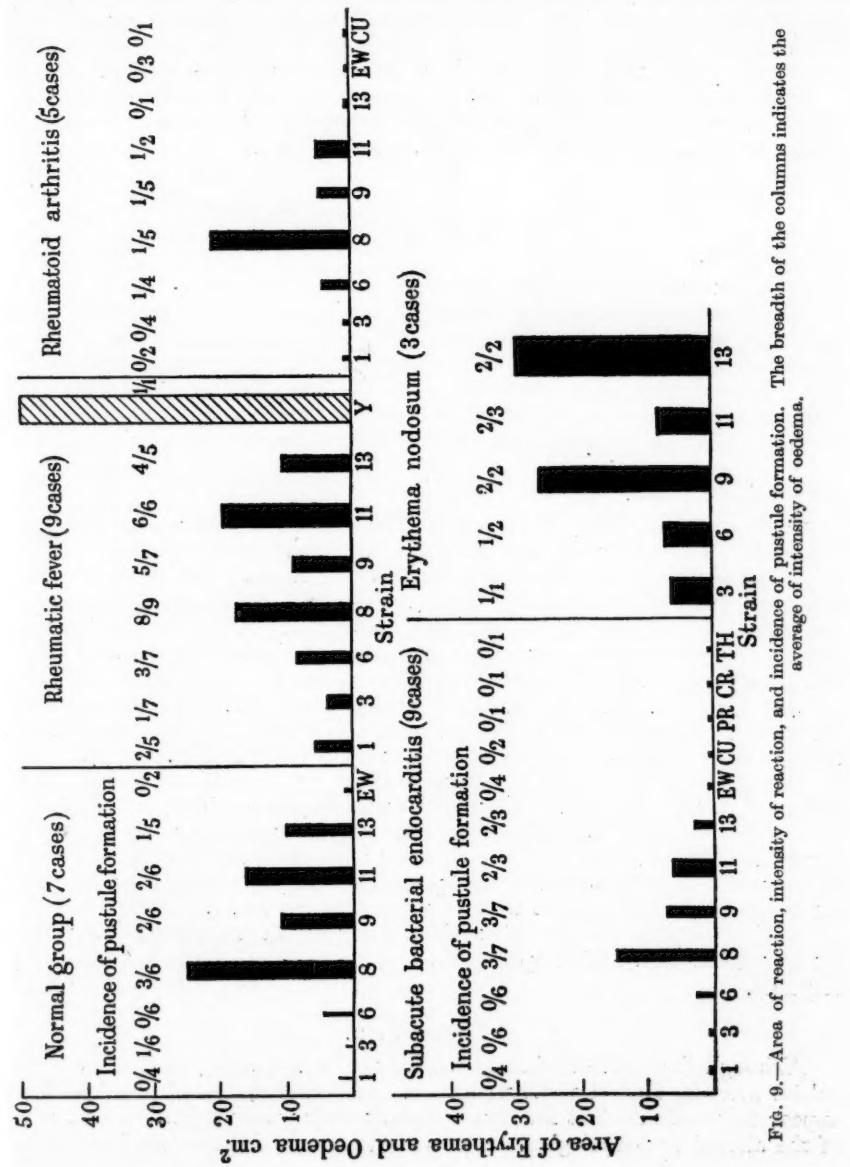
Area of Erythema and Oedema cm.²
Normal group (7 cases)
Rheumatic fever (9 cases)
Rheumatoid arthritis (5 cases)
Incidence of pustule formation
0/4 1/6 0/6 3/6 2/6 2/6 1/5 0/2 2/5 1/7 3/7 8/9 5/7 6/6 4/5 1/1 0/2 0/4 1/4 1/5 1/5 1/2 0/1 0/3 0/1
1 3 6 8 9 11 13 EW 1 3 6 8 9 11 13 Y 1 3 6 8 9 11 13 EW CU
Strain
Subacute bacterial endocarditis (9 cases)
Erythema nodosum (3 cases)
Incidence of pustule formation
0/4 0/6 0/6 3/7 3/7 2/3 2/3 0/4 0/2 0/1 0/1 0/1 1/1 1/2 2/2 2/3 2/2
1 3 6 8 9 11 13 EW CU PR CR TH 3 6 9 11 13
Strain

FIG. 9.—Area of reaction, intensity of reaction, and incidence of pustule formation. The breadth of the columns indicates the average of intensity of oedema.

scatter inside each group, and the numbers are too small for statistical analysis. It will be observed that in normal subjects the Group A strains (8, 9, 11 and 13) gave rise to moderate reactions. In the rheumatic fever group the reactions to all strains were somewhat increased, and in the erythema nodosum group they were greatly increased. The patients with rheumatoid arthritis reacted rather less intensely than the normal group, and those with subacute bacterial endocarditis gave very small reactions even with their endogenous strains.

Apart from the fact that the Group A strains gave the most intense reactions, there was no obvious correlation between the severity of the reactions and any of the known antigenic components of the streptococci.

HISTOLOGICAL FINDINGS.

Normal cases.

Six normal persons were examined.

The histological findings consisted of small perivascular and perifollicular collections of lymphocytes, a few eosinophils and macrophages, the latter containing cocci. There were also cocci lying free in the perivascular spaces. The latter were dilated and contained a network of fibrillary material. These " lymphangitic " changes appeared to be characteristic of the tissue response of normal individuals to heat-killed stock strains of streptococci (Fig. 1 and 2).

Pathological changes.

These were observed in 21 cases of 26 examined (Table I). The central change in these cases was oedema. This appeared (*a*) as a diffuse infiltration of the cutis with fluid, separating the collagen fibres. The latter were often thickened and had occasionally lost their affinity for collagen stains, while the elastic and argentaffine fibres remained unaffected. (*b*) In some cases patchy oedema was seen with an impressive swelling and vacuolation of fibrocytes, the latter forming small " nodules " containing one or more giant cells. Such changes were sometimes accompanied by deposition of fibrinoid material in the vessel walls, along the whole circumference or confined to one side only. No cocci were seen in these areas of diffuse or patchy oedema (Fig. 3 to 8).

These altered types of reaction were observed in response to one or more strains of streptococcus in all of 9 patients with rheumatic fever, in 6 of 9 patients with subacute bacterial endocarditis, in all of 3 patients with streptococcal erythema nodosum, and in 3 of 5 patients with rheumatoid arthritis (Table I). The response to other strains in these patients was of the normal " lymphangitic " types, although intermediate changes were sometimes met. There was no clear correlation between the intensity of the early erythema and oedema produced by any given strain of streptococcus and the nature of the later histological response.

DISCUSSION.

Although the number of cases studied was small, being limited by the difficulties attendant upon work with human volunteers, the histological findings appear to be of sufficient interest to justify their presentation. Tissue changes of the altered or pathological type are reminiscent of those seen in local serum

TABLE I.—*Incidence of Abnormal Histological Changes Produced by Injection into the Skin of Different Strains of Streptococcus in Different Groups of Subjects.*

Strain.	Normal subjects.	Rheumatic fever.*	Rheumatoid arthritis.	Subacute bacterial endocarditis.	Erythema nodosum.
1	0/4	1/5	0/2	0/2	..
3	0/6	3/7	0/4	2/5	1/1
6	0/6	4/8	3/5	1/6	2/2
8	0/6	6/9	3/5	3/7	3/3
9	0/6	6/7	0/5	3/7	2/2
11	0/6	5/6	1/2	0/2	..
13	0/5	2/5	0/1	0/2	..
†Y	..	†1/1	..	..	..
†Ew	0/2	..	1/2	†1/3	..
†Cu	..	..	0/1	†1/1	..
†Pr	..	..	..	†1/1	..
†Fu	..	..	..	†1/1	..
†Cr	..	..	..	†0/1	..
Number of cases showing abnormal response to one or more strains	0/6	9/9	3/5	6/9	3/3

* One patient, examined at the beginning of her illness and again a year later, has been counted as two cases.

† These strains were autogenous strains of α- or non-haemolytic streptococci.

hypersensitivity or the Arthus' phenomenon, in which extensive oedema is the most impressive structural change. When an Arthus type of response is comparatively mild oedema may be "focal"—with conglutination of fibres and cells in small patches of the dermal and subcutaneous tissue without affecting the argentaffine fibres (Pagel, 1939). It may be recalled that "early rheumatic infiltration" (Klinge, 1933), i.e. the initial change preceding the formation of the rheumatic nodule, consists of oedema leading to separation and fibrinoid impregnation of fibrils without affecting the argentaffine reticulum fibres. Undue emphasis, however, should not be laid upon a possible resemblance between the response to heat-killed streptococci and rheumatic changes.

The following appear to us to be the main points which emerge from this study: First, the late histological reaction affords an important supplementary indication of the type of sensitivity with which we are concerned—one, moreover, which is probably fairly independent of the initial direct "toxic" effects of the substances injected. Second, the "positive" groups show a sensitized type of reaction to streptococci. This was not unexpected in view of existing or preceding infection with streptococci in a number of the patients, but the variability in response to different strains is interesting. Finally, the hypersensitive response in this series is of the Arthus type, although provoked by bacterial antigens. It has usually been observed that such antigens elicit a delayed reaction, with predominant necrosis, in contrast to serum hypersensitivity, where there is a rapid reaction with predominant oedema (Rich, 1944).

SUMMARY.

A study has been made of the histological changes observable 10–18 days or more after injection of various strains of heat-killed streptococci into the skin of normal human subjects and of patients with rheumatic fever, subacute bacterial endocarditis and streptococcal erythema nodosum.

"Normal" and "hypersensitive" types of response are described, the latter having the characteristics of an Arthus-type reaction. It included focal oedema with conglutination of collagen fibres, fibrinoid infiltration of vascular walls and formation of fibrocytic nodules.

"Hypersensitive" types of response were obtained with one or more strains of streptococcus in all of 9 cases of rheumatic fever, in 6 of 9 cases of subacute bacterial endocarditis, all of 3 of erythema nodosum, and in 3 of 5 of rheumatoid arthritis but in none of 6 normal subjects.

Autogenous strains of streptococcus gave hypersensitive responses more frequently than the others, but otherwise there was no clear correlation between the possession of particular antigenic constitution and the type of histological response.

The late histological changes were not closely correlated with the short-term reactions of oedema, erythema and pustule formation.

Our warmest thanks are due to the patients, staff and students at the Central Middlesex Hospital, who were most willing volunteers, to Mr. J. Mayhew for technical and Miss Hedwig Saxl and Miss Beryl Buckle for photographic assistance.

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QUANTITATIVE STUDIES IN DIPHTHERIA PROPHYLAXIS: THE PRIMARY RESPONSE.

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For many years both qualitative and quantitative data have been published concerning the response of different animals to diphtheria toxoid, administered in different ways, in different amounts, and with different time intervals between inoculations. Since formol-toxoid and the more active adsorbed toxoids, such as alum precipitated toxoid, were themselves variable in potency (Bousfield, 1947) and prepared in different ways (Lewis, 1941), the significance of these published results could not easily be assessed.

With the introduction of a purified toxoid adsorbed on to a suspension of aluminium phosphate—P.T.A.P. (Holt, 1947)— a reagent was made available in which variations in the proportion and concentration of the two components—toxoid and carrier—could be altered at will, and reliance placed on the reproducibility in the composition and antigenicity of different preparations.

The characteristic response of an animal, say a guinea-pig, to a primary inoculation is well illustrated by experiments reported by Glenny and Sudmerson (1921). These workers inoculated a number of guinea-pigs with a single dose of underneutralized toxin-antitoxin mixture. There occurred a latent period of about three weeks, followed by the appearance of circulating antitoxin, which reached a peak value in about eight weeks, followed by a fall in titre. In one such experiment the serum antitoxin titres of guinea-pigs were followed over a period of two years, and for the greater part of the latter period of observation the animals showed a constant titre of circulating antitoxin—the final baseline level of immunity. Recently Glenny and Barr (1947) have reported a similar phenomenon in horses, in which tetanus antitoxin titres were observed over a period of five years following a single inoculation of tetanus toxoid.

Since it has been shown by Schoenheimer, Ratner, Rittenburg and Heidelberger (1942) that the "half-life" of a globulin molecule is two weeks, the prolonged maintenance of circulating antitoxin clearly indicates a continuous production of antitoxin by the globulin-producing cells. This conclusion is in keeping with that of Burnet, Freeman, Jackson and Lush (1941): "it is equally conceivable that once the antigenic stimulus has been applied, the cells continue to produce antibody long after the antigen has been completely disintegrated." Glenny and Barr (1949) have expressed very similar conclusions. It also shows that the capacity to produce the altered globulin, antitoxin, as a result of an inoculation of toxin or toxoid presumably depends on a permanent alteration, or kainogenetic effect, in the pattern of the globulin elaborated by these cells. Since, also, the circulating antitoxin persists for at least two years in guinea-pigs and five in horses, the original stimulus must have induced so profound a modifica-

tion in the globulin-producing cells that its effect was handed down to daughter cells, for it is unlikely that the original antibody-producing cells would survive for so long. In other words, it is suggested that the change brought about by the action of toxin or toxoid on the globulin-producing cells is one that affects the very genetics of the cells.

It follows from the above considerations that a primary inoculation of toxoid stimulates a number of globulin-producing cells, which subsequently elaborate antitoxin instead of normal globulin, and continue to do so at an even tempo for a substantial period of time. The shape, therefore, of a pure primary response curve would be as drawn in Fig. 1.

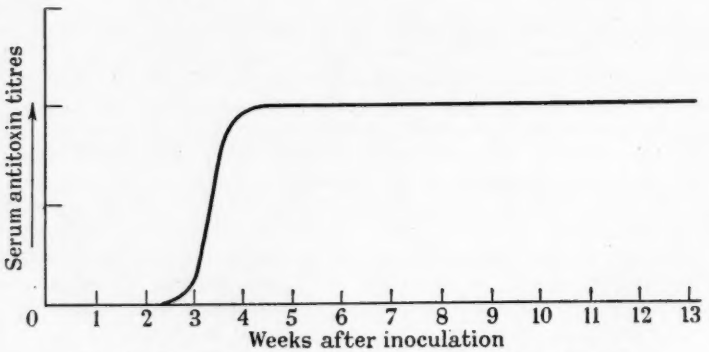

FIG. 1.—Theoretical pure primary response to diptheria toxoid.

Can such an ideal primary response curve be obtained ?

When formol-toxoid is employed as a primary stimulus, and the response closely followed, we may obtain one of two differently shaped curves, shown in Fig. 2. The time for maximum response in the curve showing a marked decline (B) is usually 6–7 weeks, whereas for the one rising to an almost constant level (A) it is 4 weeks. The latter closely approximates the theoretical curve. It is suggested that Curve B is another example of the marked effect of the presence of traces of toxin in toxoid on the short-range antitoxin response when used as a primary stimulus (Glenny, Pope, Waddington and Wallace, 1925). This, perhaps, owing to local fixation of the toxin in the non-immune animal, with subsequent release, would act as a *second stimulus*.

Formol-toxoid has been largely displaced by alum-precipitated toxoid (A.P.T.), which was shown by Glenny, Buttle and Stevens (1931) to be some one hundred times as potent. They ascribed this increased antigenic efficiency to a resulting slow adsorption and elimination of the precipitated toxoid.

Recently the antitoxin-time-response curves have been observed in groups of not less than 12 guinea-pigs, which had received subcutaneous inoculations of 1 Lf amounts of purified toxoid adsorbed on to different quantities of pure aluminium phosphate carrier (Fig. 3).

In all cases the time required to reach maximum mean antitoxin titre was the same (about 25 days), but the final base-line mean titres were different, being

higher as the amount of $AlPO_4$ carrier injected was increased up to the optimal amount of 3 mg. (Holt, 1947).

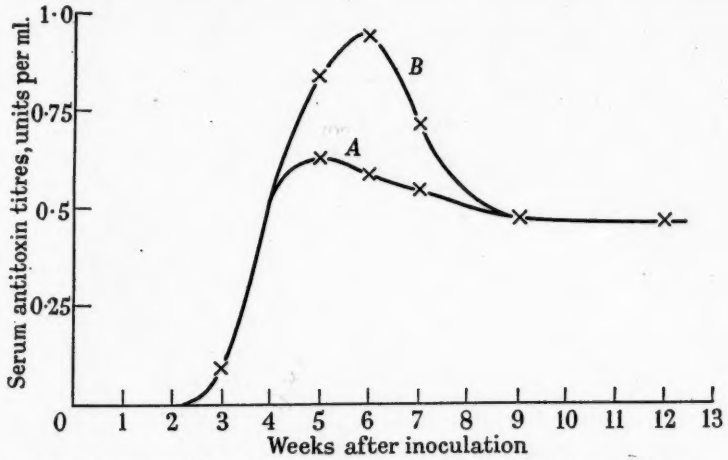

FIG. 2.—Types of primary response to diptheria formol-toxoid, e.g. 100 Lf.

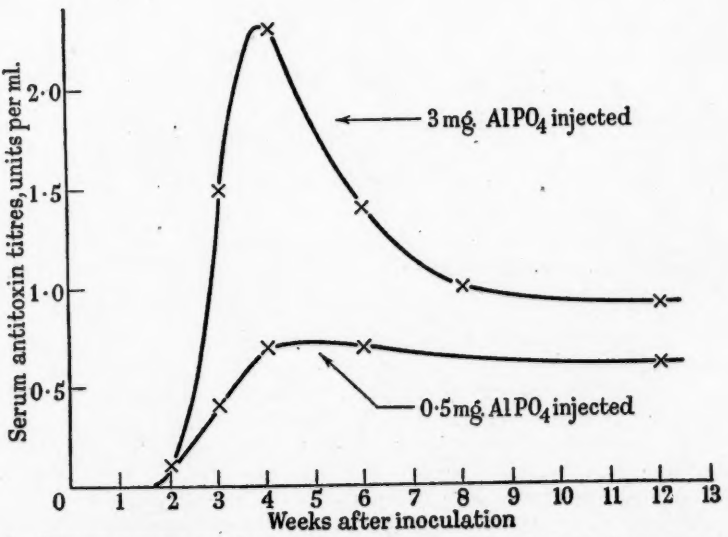

FIG. 3.—Primary response to subcutaneous inoculation of P.T.A.P. (1 Lf; dose vol. 0·5 ml.)

When the nodules which develop after the injection of 3 mg. of $AlPO_4$ carrier (with 1 Lf of toxoid) were surgically excised at different times after inoculation, and the antitoxin response curves again followed, it was found that there was no

significant difference in the curves when the interval between injection and excision exceeded 14 days. But when the nodules were excised 7 to 10 days after inoculation a peak titre did not occur; instead a response was obtained almost identical with the theoretical pure primary (Fig. 4). This same type of response was also observed when the carrier disappeared completely from the site of inoculation by natural means within 7 days, as is shown in Fig. 3, where the response from an injection of 0·5 mg. of carrier was followed.

It is important to note that the levels of circulating antitoxin at 12 weeks (Fig. 4) were the same whether the nodules (3 mg. $AlPO_4$ injected) were excised

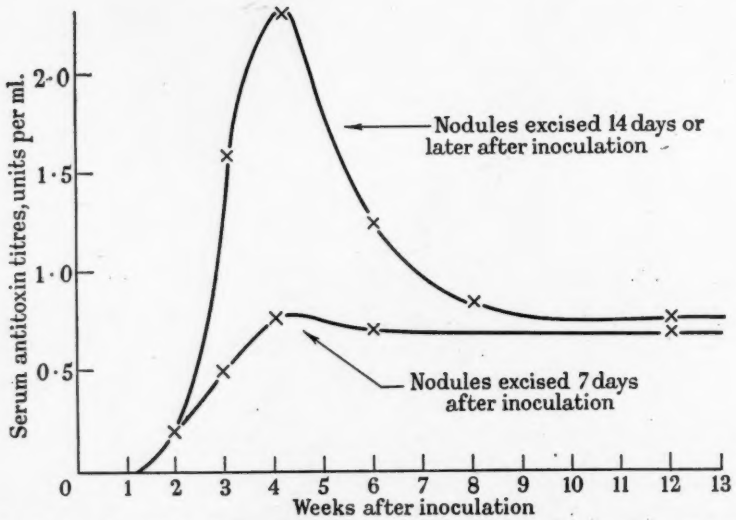
Serum antitoxin titres, units per ml.
2·0
1·5
1·0
0·5
Nodules excised 14 days or later after inoculation
Nodules excised 7 days after inoculation
0 1 2 3 4 5 6 7 8 9 10 11 12 13
Weeks after inoculation

FIG. 4.—Effect of excision of subcutaneous nodule on the antitoxin response. The subcutaneous nodules were exercised 1, 2, 3, 4, and 6 weeks after inoculation; controls no excisions. The responses were virtually identical in all cases except that where the nodules were excised after 7 days.

after 7 days, 6 weeks, or left *in situ*. When, however, the nodules were excised before the 7th day after injection, the response was less the earlier they were removed, since the shorter the time interval, the less material is transported to the antibody-producing cells (*vide infra*).

The difference, therefore, between the responses obtained when the nodules were excised between 7 and 10 days after inoculation and those left *in situ* represents a second response superimposed on the pure primary. The immunological significance of the nodule remaining *in situ* much longer than one week is of small practical importance, since the final base-line level of immunity, i.e. circulating antitoxin, is unaltered by its presence. The significant time interval is the first 7 to 10 days.

In addition, these findings show that a second response may be obtained as early as 10 to 14 days after a primary inoculation.

An examination of the *cellular* response in guinea-pigs to moderate amounts of A.P.T. or P.T.A.P. injected subcutaneously showed that in some 6 hours leucocytic infiltration had commenced, and was well marked in 24 hours. Also, particles of carrier could be demonstrated in the granulocytes. This was done by "staining" sections of the subcutaneous nodules with alumon (Aurine-tricarboxylic acid). Subsequently the mass enlarges by leucocytic infiltration, becomes rounded-off and enclosed in a fibrous capsule, and the contained granulocytes then show karyorrhexis. The process of encapsulation requires 7 to 10 days (Fig. 7). Then the mass contracts and the capsule thickens by increased fibrosis.

When the nodules were excised *intact* 7 to 10 days after inoculation, and implanted subcutaneously into sensitized guinea-pigs, there followed only a

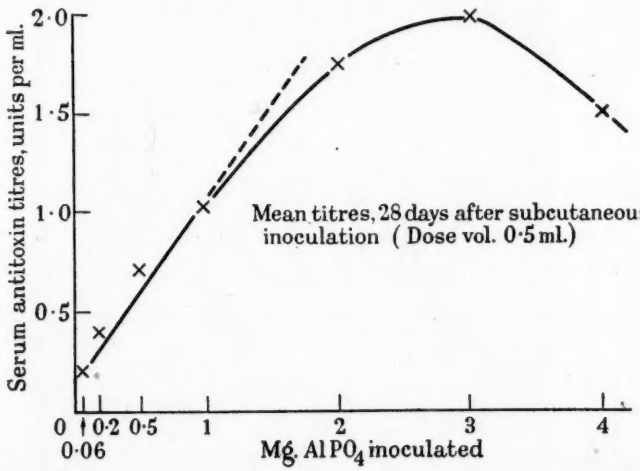

FIG. 5.—Influence of amount of $AlPO_4$ inoculated on the antitoxin response. (Dose toxoid 1 Lf.)

very small second response, which may well have resulted from a slight rupturing of the delicate young capsule during the necessary surgical manipulations. Such 10-day-old nodules, after being macerated, gave rise to a marked second response when inoculated into sensitized guinea-pigs.

Since it has now been shown that when the residual mass of adsorbed-toxoid is excised between 7 and 10 days after subcutaneous inoculation the antitoxin response is almost identical with the theoretical primary one, and produces the same final base-line level of circulating antitoxin as a non-excised inoculum, and that fibrous encapsulation of the residual mass is complete in about the same time, it is concluded that neither the second response phenomenon, nor delayed adsorption of the antigen (depot hypothesis of Glenny, Buttle and Stevens, 1931), is responsible for the enhanced antigenicity of adsorbed toxoid when used as a primary stimulus.

A comparison has been made of the antigenic efficiency of samples of P.T.A.P. containing different amounts of $AlPO_4$ carrier and employing both the sub-

cutaneous and intramuscular routes. When 2·5 mg. $AlPO_4$ in P.T.A.P. was inoculated subcutaneously in young children, the incidence of palpable nodules was 90 per cent, and 100 per cent when larger amounts were inoculated (Bousfield, 1947). The same amounts of $AlPO_4$ injected intramuscularly invariably produced the better Schick conversion rates (Holt and Bousfield, 1949). It is suggested, therefore, that the reason for the increased Schick conversion rate among those children which received the P.T.A.P. inoculations intramuscularly is that the continuous movement of the muscle delayed or even prevented fibrous encapsulation of much of the material injected, although this is not readily demonstrable. This would result in a greater fraction of the injected mass reaching the antitoxin-producing cells.

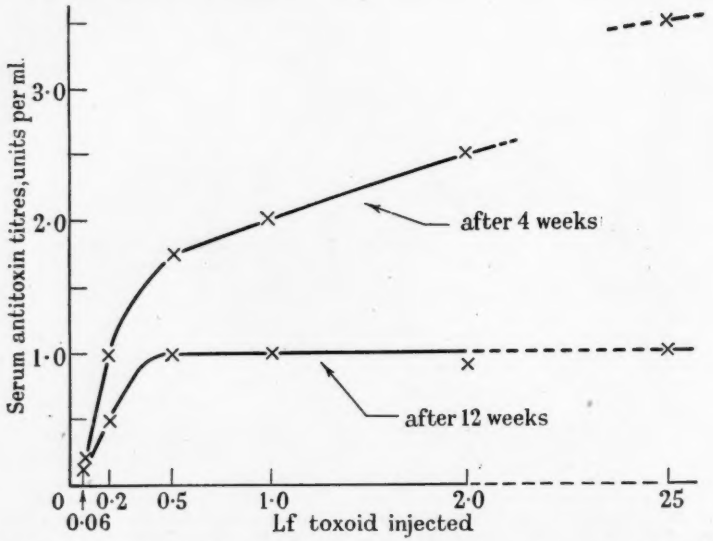

FIG. 6.—Antitoxin response, in guinea-pigs, $AlPO_4$, carrier constant at 3 mg. Injected. Dose of toxoid varied.

It is suggested that the reason for the enhanced antigenicity of A.P.T. and P.T.A.P. is that the mineral carrier conditions the amount of toxoid that reaches the antitoxin-producing cells via the granulocytes.

An examination of the cellular response in guinea-pigs to subcutaneous injections of aluminium phosphate greater than 2·5 mg. revealed that, instead of complete and thorough infiltration of the mass by leucocytes as described earlier for smaller amounts, the granulocytes had succeeded only in penetrating the periphery of the mass.

When the injection volume is 0·5 ml. and the injections are made subcutaneously on the ventral wall, the guinea-pig can remove completely, without a residual nodule, slightly less than 1 mg. of aluminium phosphate. Quantities greater than 1 mg. invariably give rise to the development of well-defined subcutaneous nodules.

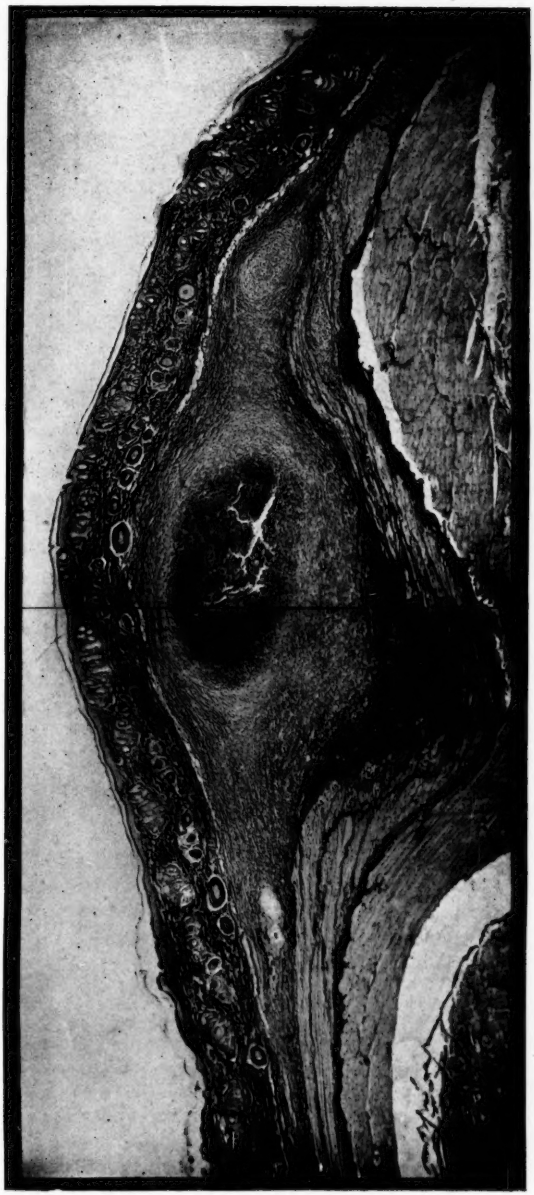

FIG. 7.—Section of guinea-pig skin, 10 days after subcutaneous inoculation of 0·5 c.c. A.P.T. (Approximately 2 mg. of mineral injected).
Note: Fibrous encapsulation of residue of injected material—the formation or the 'nodule.'

The antitoxin responses in guinea-pigs, 28 days after the subcutaneous inoculation of a constant amount of purified toxoid (1·0 Lf) adsorbed on to increasing amounts of $AlPO_4$ carrier, showed that, with amounts up to 1 mg., the response was directly proportional to the amount of carrier used. When between 1 and 3 mg. were injected the slope was less steep, and progressively larger nodules formed. The injection of more than 3 mg. of $AlPO_4$ carrier (in 0·5 ml.) resulted in a submaximal antitoxin response (Holt, 1947). Although much of the higher values was due to a second response effect (Fig. 3 and 4), the 3 mg. inoculation gave the highest base-line level of circulating antitoxin. It is suggested that, in fact, more carrier is phagocytosed when 3 mg. is injected subcutaneously than when greater or less amounts are used, 3 mg. being the point of maximum advantage in respect of two opposing forces—amount of carrier to be phagocytosed and loss of carrier by encapsulation.

If this interpretation is correct, viz. the antitoxin response to samples of P.T.A.P. is conditioned by the number of particles of carrier which, with their charge of adsorbed toxoid, are phagocytosed and escape to reach the globulin-producing cells, the limit being the receptive capacity of the animal employed, then the maximum response to an inoculation of 1 Lf of toxoid would only be obtained when the toxoid is distributed among the globulin-producing cells in such a way that each cell that receives toxoid receives one minimal stimulating dose.

An experiment was carried out employing different amounts of purified toxoid, adsorbed on to 3 mg. of $AlPO_4$, the amount of carrier which had been found to be optimal for 1 Lf of toxoid. Within the limits of 0·5 Lf and 25 Lf there was little difference in the antitoxin responses in guinea-pigs 4 weeks after injection, and *none* after 3 months. The antitoxin response declined rapidly as amounts less than 0·5 Lf were used (Fig. 6).

It is suggested that when 0·5 Lf of toxoid is adsorbed on to 3 mg. of $AlPO_4$ the " statistical " particle, viz. that quantity of $AlPO_4$ that reaches one globulin-producing cell, carries just one minimal stimulating dose of toxoid. When less toxoid is employed, the antitoxin-producing cells fail to receive one minimal stimulating dose. When, however, quantities of toxoid greater than 0·5 Lf/3 mg. $AlPO_4$ are used, each antitoxin-producing cell would receive an excess of toxoid. If one assumes that the " All or None Law "—as applies to the heart and nerves—also holds for the reactivity of antitoxin-producing cells, then those cells which receive an excess of antigen will make no greater response than those which receive one minimal stimulating dose. On the other hand, summation of subminimal doses may result in a response if the time interval between the several stimuli is not too prolonged.

The ratio 0·5 Lf/3 mg. $AlPO_4$, or 0·16 Lf/mg. $AlPO_4$, is therefore the critical ratio of toxoid to $AlPO_4$ carrier, for maximal antigenic efficiency in guinea-pigs.

The results of a clinical investigation on similar lines has recently been reported (Holt and Bousfield, 1949).

In this inquiry Schick-positive young children were inoculated intramuscularly with samples of P.T.A.P., the dose of $AlPO_4$ carrier was constant at 7·5 mg., but the amount of purified toxoid used varied. The Schick conversion rates, determined 6 months later, showed no statistically significant difference in the results, whether 5 Lf or 30 Lf of toxoid were injected with the 7·5 mg. of $AlPO_4$—limits 95·2 to 98·4 per cent Schick conversion, 634 cases examined.

The lowest toxoid to carrier ratio employed was 0·66 Lf/mg. $AlPO_4$. Efforts to determine the " human " minimal effective ratio of toxoid to carrier are now in progress, and it will be interesting to learn how closely it agrees with the guinea-pig ratio.

Since 25 Lf of formol-toxoid will give rise to about 0·1 u/ml. serum antitoxin in guinea-pigs, and this represents a moderate response to the antigen in simple solution, and 0·5 Lf adsorbed on to 3 mg. $AlPO_4$ induces 1·0 u/ml. which represents the response using the optimal amount of carrier, in so far as these figures are comparable it may be calculated that the antigenicity of the toxoid has been increased 500 times by adsorption in the proportion given :

$$\text{Ratio} = \frac{25 \times 1{\cdot}0}{0{\cdot}1 \times 0{\cdot}5}.$$

SUMMARY.

The shape of a pure primary response curve to an inoculation of toxoid is deduced.

This pure primary response may be obtained with adsorbed toxoid, by suitably timed surgical excision of the material inoculated.

It is shown that the enhanced potency of adsorbed toxoid is not due to the second response phenomenon, nor to delayed release from a depot, but to a more efficient distribution of the toxoid among the antitoxin-producing cells. This, it is suggested, is brought about by the *transportation* of the (adsorbed) antigen to the antitoxin-producing cells by the polymorphonuclear leucocytes which have been shown actively to phagocytose the $AlPO_4$ carrier used.

The critical minimal ratio of toxoid to $AlPO_4$ carrier for the immunization of guinea-pigs has been determinated and found to be approximately 0·16 Lf/mg. $AlPO_4$. By comparing the antitoxin response in guinea-pigs to 25 Lf of formol-toxoid with that obtained with 0·5 Lf adsorbed on to the optimal amount of carrier (3 mg. $AlPO_4$), the maximum ratio of antigenic efficiency of adsorbed to its parent formol-toxoid, when used as primary stimuli, has been calculated to be of the order of 500.

The second response phenomenon may be elicited as early as 10–14 days after primary inoculation.

It was suggested by Burnet *et al.* (1941) that once the antigenic stimulus has been applied, the antibody-producing cells continue to produce antibody long after the antigen has been disintegrated.

It is now suggested, in respect of diphtheria antitoxin production, that this kainogenetic effect is inherited by the *daughter cells* of those originally stimulated.

Grateful thanks are due to Dr. W. W. Wilson, of the Department of Morbid Anatomy, for the guinea-pig skin sections, and to Mr. W. Pereira for the photomicrograph.

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STUDIES ON STAPHYLOCOCCUS MUTATION: AN INVESTIGATION OF THE GROWTH REQUIREMENTS OF A "G" (GONIDIAL) VARIANT.

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IN a previous communication (Hale, 1947) I suggested that the small colony size of the so-called "G" gonidial variants of the staphylococcus results from its requiring growth factors in excess of the needs of the parent strain. This present paper reports the results of an investigation of the growth requirements of one of these variants, selected by the use of gentian violet.

METHODS.

The strains used in this investigation were:

Parent Strain Au 18L—isolated from an abscess.

Variant Au 18S—isolated from Au 18L by the aid of gentian violet (Hale, 1947).

Tubes for testing growth requirements received 4·5 ml. of the basic media + 0·5 ml. of a dilution of the substance under test, or 0·5 ml. saline in the case of the control tubes.

For the preparation of an inoculum the organisms were grown for 24 hours at 37° C. on nutrient agar slopes. Suspensions were made in saline, enumerated turbidometrically and dilutions made so that 1 ml. contained 5×10^3 cocci per ml. Each tube then received 0·2 ml. of this diluted suspension to give an inoculum of 1000 cocci or 200 cocci per ml. media.

EXPERIMENTAL.

Preliminary investigation of the growth of the gonidial variant.

The commencement of growth of the variant Au 18S was delayed in nutrient broth and the final population attained was always much smaller than that of

the parent strain Au 18L. Nevertheless, it was decided for this investigation to use a simple medium that, while allowing normal growth of the parent strain, would not support, or at best give only minimal, growth of the variant. Such a medium could then be supplemented so that it gave a growth of the variant comparable to that of the parent. Analysis of these supplements should then reveal the additional factors required for maximal growth by the variant.

Casein hydrolysate medium prepared according to McIlwain and Hughes (1944), providing it was supplemented by glucose, proved to be adequate for this purpose.

TABLE I.—*Growth of Staphylococci in Casein Hydrolysate Basal Medium.*

		Incubation in hours.				
		17.	24.	41.	48.	66.
Casein Hydrolysate	Au 18L	−	±	+	++	++
	Au 18S	−	−	−	−	?
Casein Hydrolysate + Glucose M/50	Au 18L	±	+++	++++	+++	++++
	Au 18S	−	−	tr.	tr.	±

NOTE.—In this and all subsequent tables: + signs indicate degrees of growth. tr. = trace of growth.

Sources of growth factors necessary for variant strain.

Yeast extract was prepared by crumbling 1 lb. of bakers' yeast into 1 litre of water at 85° C. and the whole was held at that temperature for 15 min. After paper filtration to remove yeast cells the filtrate was sterilized by Seitz filtration and tested by addition to the basic casein medium.

The results show that this material added to the basic medium supported a growth of the variant equivalent to that of the parent after 66 hours' incubation at 37° C. This activity was exhibited at 1/10 final dilution of the extract and some growth stimulation was noticed in higher dilutions. Precipitation of the yeast extract by lead acetate left the active material in the filtrate.

Fresh human serum and human urine freshly voided and sterilized by Seitz filtration were also found to exhibit similar activity to yeast extract in promoting maximal growth of the variant in both cases at final dilution of 1/10.

TABLE II.—*The Effect of Yeast Extract, Serum and Urine on Growth.*

	Strain.	Final dilutions of substance tested. 10^{-1}.	10^{-2}.	10^{-3}.	Control.	Incubation time.
Yeast Extract	Au 18L	++++	++++	++++	++++	66 hr.
	Au 18S	++++	++	+	±	
Serum	Au 18L	++++	++++	++++	++++	58 „
	Au 18S	+++	++	tr.	±	
Urine	Au 18L	++++	++++	++++	++++	50 „
	Au 18S	++++	++	±	tr.	

Medium = Casein Hydrolysate + M/50 Glucose.

Marmite, obtained from the manufacturers free of salt and made up to a 30 per cent w/v solution, proved to be highly active. It gave growth of the variant comparable with that of the parent up to a final dilution of 1/500 and demonstrated considerable growth stimulation up to a dilution of 1/62,500.

Further investigations demonstrated that the active material was extractable by acids, but not by ether, chloroform or amyl alcohol.

TABLE III.—*The Effect of Marmite* (30 *per cent w/v Solution*) *on Growth.*

	Marmite dilutions in basal medium.	Incubation in hours. 24.	48.
Au 18L	10^{-1}	++++	++++
	10^{-2}	++++	++++
	1/500	++++	++++
	1/2,500	++++	++++
	1/12,500	++++	++++
	1/62,500	++++	++++
	C	+++	++++
Au 18S	10^{-1}	+++	++++
	10^{-2}	++	++++
	1/500	±	++++
	1/2,500	±	+++
	1/12,500	tr.	+++
	1/62,500	tr.	++
	C	tr.	+

Using casein hydrolysate medium it was found that the control tubes containing no supplementary factors did show minimal growth after prolonged incubation, and at this stage it was thought better to substitute for this medium the synthetic amino acid medium described by Fildes, Richardson, Knight and Gladstone (1936) and modified by Fildes and Richardson (1937) and Gladstone (1937). With this medium the above results were repeated, and providing the inoculum did not exceed 200 cocci per ml. of medium the control tubes showed no visible growth after 7 days' incubation, although from these control tubes the presence of viable organisms could usually be demonstrated.

The effect of yeast nucleic acid on the growth of the variant.

The distribution and certain properties of the active material suggested the testing of yeast nucleic acid. Yeast nucleic acid was prepared according to the method described by Cole (1926) and a 5 per cent w/v solution was active in stimulating growth up to a dilution of 1/100.

The result suggested the use of yeast nucleic acid hydrolysed under different conditions to give in the hydrolysed mixtures predominantly nucleotides, nucleosides or the free bases. Hydrolysis was carried out under the conditions described by Levene and Bass (1931). The hydrolysed mixture that contained the free pyrimidine and purine bases was a hundred times more active than either the original yeast nucleic acid or the mixtures that contained nucleotides or nucleosides. This difference was so marked that it appeared most probable that the slight growth-promoting activity of the yeast nucleic acid and the other mixtures was, in fact, due to the presence of free bases in these materials.

Uracil as a "growth stimulator" of the variant.

A test carried out using the individual bases known to be present in yeast nucleic acid demonstrated quite clearly that, while uracil was active in promoting growth up to a concentration of 10^{-6} M, guanine, cysteine and adenine were ineffective.

TABLE IV.—*The Effect of Hydrolysis on the Growth-stimulating Properties of Yeast Nucleic Acid.*

	Final dilutions of substance tested.				
	10^{-1}.	10^{-2}.	10^{-3}.	10^{-4}.	Control.
Yeast nucleic acid	++++	++++	++	−	−
Yeast nucleic acid hydrolysed with weak ammonia at 115° C. (nucleotides)	++++	++++	+++	−	−
Yeast nucleic acid hydrolysed with 20 per cent ammonia at 150° C. for 3½ hours (nucleosides)	±	++++	++	±	−
Yeast nucleic acid hydrolysed with 25 per cent H_2SO_4 2 hours (free bases)	?	++++	++++	++++	−

Test organism is the Variant Strain Au 18S.
Synthetic medium 7 days' incubation.
The yeast nucleic acid solution was 5 per cent w/v and the hydrolysed mixture arranged so that 5 g. of yeast nucleic acid was hydrolysed and then made up to 100 ml.

TABLE V.—*The Effect of Uracil on the Growth of the Variant.*

	Final dilutions of substance tested.					
	10^{-4} M.	10^{-5} M.	10^{-6} M.	10^{-7} M.	10^{-8} M.	Control.
Uracil	++++	++++	++++	+	±	±
Adenine	+	±	±	..	..	±
Cytosine	±	±	±	..	..	±
Guanine	±	±	±	..	..	±

Synthetic Medium, 7 days' incubation.

DISCUSSION.

The results reported above prove that the growth of the small variant, selected by the aid of gentian violet from cultures of the parent staphylococcus Au 18L, is stimulated by the addition of uracil to the chemically defined medium described by Fildes and Richardson (1937) and Gladstone (1937). Uracil is necessary for the growth of staphylococci under anaerobic conditions (Richardson, 1936) but aerobically they are apparently able to synthesize sufficient uracil for their requirements. The variant strain reported here differs in that the aerobic synthesis of uracil is also very much impaired. This aerobic synthesis is not completely suppressed as an inoculum of the variant in the synthetic medium does grow eventually (after 7 days). Growth of the parent strain and the variant under anaerobic conditions, when both will be dependent upon the uracil present in the medium, should therefore approximate. This is found to be the case, for nutrient agar plates of the parent and variant incubated under anaerobic conditions exhibit colonies approximately equal in size.

The impaired synthesis of uracil by the variant must result in deficiency of ribo-nucleic acid. Henry and Stacey (1943) have shown that the staining properties of Gram-positive organisms depend upon the presence of magnesium ribo-nucleate; therefore it is not unreasonable to assume that the staining properties of the variant might differ from those of the parent. It was found that when the organisms were stained with a mixture of an acid and basic dye

the parent failed to take the acid dye until a pH of 2·0 was attained, whereas the variant changed over at pH 3·0–3·5. This is more characteristic of Gram-negative organisms where changeover takes place at pH 4·0–4·5. Gram-negative organisms are more resistant to gentian violet than gram-positive organisms (Stearn, 1927), probably because they have not the same affinity for this basic compound. By the same reasoning one could account for the variant's greater resistance to gentian violet and its consequent selection in cultures containing concentrations of gentian violet just inhibitory to the parent strain.

It is not claimed that uracil is the only requirement by which the variant differs from the parent. Thus, addition of uracil to solid media does definitely stimulate the growth of the variant, but the colonies are still not as large as those of the parent on the same media less uracil. Other substances were found which stimulated the growth of the small variant, but only if uracil was present. It appears, therefore, that uracil is the most important requirement.

SUMMARY.

(1) A small colony variant of staphylococcus was isolated by cultivation on media containing gentian violet.

(2) The growth characteristics of the variant were shown to be dependent upon an impairment of uracil synthesis. Addition of uracil to the medium stimulated growth.

(3) The selective action of gentian violet in relation to uracil metabolism is discussed.

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The author also wishes to express his indebtedness to Professor Wilson Smith for constant advice and criticism.

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A RAPID CONCENTRATING STILL.

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IN the apparatus described here aqueous solutions can be concentrated at rates of 12–14 l./hr. at temperatures not exceeding 36° C. Under these conditions many enzymes, bacterial toxins and other substances can be recovered in high yield. No novelty is claimed for this apparatus; it is a laboratory application of the climbing film still used in industry, and it is closely modelled on an apparatus described by Mitchell, Shildneck and Dustin (1944). See also Kemmerer (1945), Ames (1946), Muirhead (1946) and Reavell (1946*a*, *b*). The apparatus is described here because it can be made from parts (mainly standard) supplied by a British firm, and because it is thought that a detailed description of its operation would be useful to those concerned with the isolation of small amounts of biologically active substances from large volumes of solution.

Construction.

The glass part of the apparatus (Fig. 1) is constructed of "Pyrex" industrial glassware supplied by Messrs. James A. Jobling & Co., Ltd., of Sunderland (their Drawing No. 7338/2). *A* is a standard 1 in. Y-piece. (All dimensions are internal.) *B* is a standard Davies type double wall condenser, 35 in. long, with pressed ends of 1 in. bore; it is used as heat exchanger, and steam is passed through the inner and outer water jackets. The heating surface has an area of 235 sq. in. *C* is a standard 1 in. 90° elbow with a $\frac{5}{16}$ in. side tube for holding a thermometer. *D* was especially made from a 5 l. flask with a $1\frac{1}{2}$ in. top neck, a 1 in. bottom neck, and a 1 in. neck entering horizontally at a tangent with its centre 1 in. above the middle of the flask. *E* is a 20 in. length of 1 in. pipe line. *F* is a standard 1 in. T-joint. *G* is a standard 1 in. 45° elbow with a $\frac{5}{16}$ in. side tube; this side tube is generally sealed with a rubber bung. *H* is a $7\frac{7}{8}$ in. length of 1 in. pipe line. *I* is a standard $1\frac{1}{2}$ in. 90° elbow. *J* was especially made from a 1 l. flask with a 2 in. top neck, a 1 in. bottom neck, and a $1\frac{1}{2}$ in. neck entering the flask horizontally at a tangent in the middle. *K* was especially made from $\frac{3}{8}$ in. tube with pressed ends of 1 in. bore; for ease in assembly the vertical part of it is cut into three equal lengths, which are then connected with rubber tubing (not shown on the diagram). *L* is a standard 2 in. U-bend. *M* is a standard 3 in. to 2 in. reducer. *N* is a standard 42 in. length of 3 in. pipe line. All the parts listed above are held together in the usual way by bolts passing through metal joint flanges fitted with graphited asbestos inserts; rubber interface joint gaskets are placed between each pair of pressed ends.

A copper condenser, obtainable from T. J. Maskell, 30A, Jericho Street, Oxford, drawn in Fig. 2 on four times the scale of Fig. 1, is inserted in tube *N*. It consists of four 42 in. copper tubes of $2\frac{1}{2}$ in., 2 in., $1\frac{1}{2}$ in. and 1 in. diameter

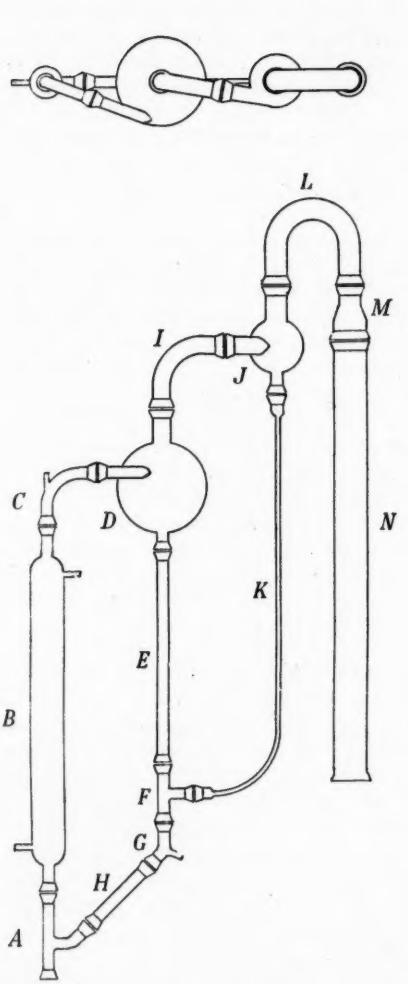

Fig. 1.

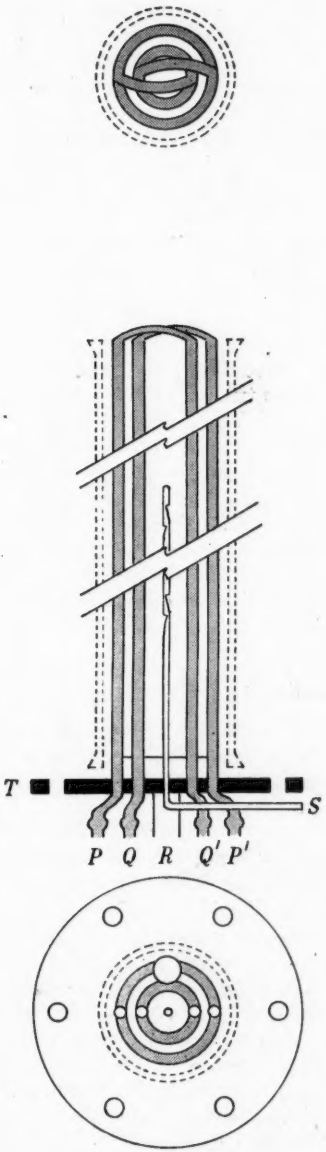

Fig. 2.

arranged concentrically to give four cooling surfaces with a combined area of 920 sq. in. The top and bottom ends of adjacent pairs of tubes are turned towards each other and welded together to form two hollow-walled cylinders which are connected together at the top, as shown, by two welded-in lengths of $\frac{1}{4}$ in. copper tubing. At the bottom ends the hollow walls of these cylinders are connected to the water supply by $\frac{1}{4}$ in. copper Tubes *PP'* and to the drain by Tubes *QQ'*. Cold water can be passed through the hollow walls of the two cylinders at a considerable rate and very efficient condensing can thus be attained. These tubes, and Tubes *R* and *S*, pass through, and are welded on to, a $6\frac{1}{2}$ in. diameter $\frac{1}{4}$ in. brass Disc *T*.

R is a $\frac{1}{2}$ in. brass tube connected by means of a Y-piece to two efficient water jet pumps. The condensate is discharged via this tube and the pumps into the drain. *S* is a $\frac{3}{16}$ in. copper tube reaching about 18 in. into the lumen of the condenser; it is perforated near the top at the condenser end, and at the other end it is connected to a mercury manometer and a vacuum release valve. A rubber interface joint gasket is placed between the end of *N* and the top of *T*, and these two parts are bolted together in the usual way.

A well-fitting rubber bung is inserted in the bottom of the Y-piece *A*, and through this bung passes the upper limb of a + piece of $\frac{5}{16}$ in. glass tubing. The lower limb and the left-hand limb are fitted with $\frac{1}{4}$ in. taps, and the right-hand limb is connected by means of a heavy wall capillary tube fitted with a tap to a reservoir of antifrothing agent. The liquid to be concentrated enters the apparatus through the tap in the left-hand limb, and the concentrate is discharged through the tap in the bottom limb. Before passing into the apparatus the dilute liquor passes through a "Rotameter" flow-meter calibrated for water flowing at rates from 6–16 l./hr.

Operation.

The water jet pumps are turned on and the dilute liquor is drawn up into the apparatus to a level about 3 in. below the bottom of Bowl *D*. The inlet tap is then turned off, the pressure is brought down to 15–20 mm. Hg., and steam issuing from a valve at about 7 lb. pressure is allowed to pass freely through the inner and outer jackets of the heat exchanger *B*. The liquid in *B* immediately starts distilling, and the vapour thus formed forces liquid into Bowl *D* at great speed. Here it is subjected to centrifugal force which separates the vapour from the liquid; the latter returns to the heat exchanger via *E F G H A* while the vapour passes to the second centrifugal separator *J*, where any residual liquor is returned to the heat exchanger through *K*. From *J* the vapour passes through *L* to be rapidly condensed in *N*. After about a minute the inlet tap is opened enough to allow the dilute liquor to enter the apparatus at about the same rate as water is being distilled off. With tapwater at about 15° C. the rate of distillation is 12–14 l./hr., and the temperature of the liquid being distilled (as measured with the thermometer in the elbow *C*) does not rise above 36° C. It is important to judge the correct rate of inflow of the dilute liquor, and for this purpose the flowmeter is a great advantage. If the dilute liquor is fed in too quickly the rapidly rotating current of water vapour will carry liquid up into the elbow *I* and constrict it and thus slow down the rate of evaporation. If the dilute liquor is fed in too slowly for a long time there is danger that the con-

centrate will dry in the heat exchanger. When the apparatus was first constructed the parts *E* and *F* were reversed so that the return arm *K* came in at a higher level, as it does in the apparatus of Mitchell *et al.* (1944). With this arrangement there was not a sufficient hydrostatic head to keep an unbroken column of liquid in *K*. The result of this was that vapour rushed through *K*, carrying liquid with it and splashing it up into *L*, whence it was carried over to the condenser by the stream of water vapour. This led to considerable " creep " loss. With the present arrangement no such difficulty is met.

The vapour from *D* enters *J* at tremendous speed—about 150 l. per sec.—which means that it passes through the 1½ in. elbow *I* at 280 miles per hour. The effect of this was seen when, for reasons now irrelevant, a heavy glass stopper was placed in the bottom of Bowl *J*. As soon as distillation started the stream of vapour lifted up the stopper and whirled it around at great velocity for a fraction of a second before hurling it through the side of the bowl.

The centrifugal action to which the distilling liquid is subjected in Bowl *D* breaks down considerable froth, but with very violently frothing liquids it is advisable to introduce 0·5 ml. of an anti-frothing agent, preferably a non-volatile one such as triamylcitrate, at the beginning of the distillation.

A given volume of liquid can be concentrated down to about 750 ml. provided the concentration of total solids is not high enough to render the concentrate unmanageably viscous. When dealing with volumes of the order of 100 l. (*ca.* 0·2 per cent total solids), or with liquids containing high concentrations of solids, it might be advisable continuously to draw off the concentrate through the side tube on *S*. This could be done by attaching a tap and a flask evacuated to a higher degree than the rest of the apparatus. This procedure has not been tried out by the author. It is probably more convenient to stop the distillation at suitable intervals and draw off the concentrate.

Distillation is stopped by first turning off the steam ; after about five minutes, when the heat exchanger has cooled down, the vacuum is released, the water jet pumps are turned off and the concentrate is drawn off. The apparatus can be cleaned as follows : Draw in water up to about half-way in Bowl *D* and reduce the pressure to about 60 mm. Hg. Then open the inlet tap. This results in a violent swirling motion of the wash water, which scours the walls of the apparatus. This is repeated several times with changes of water, and, if necessary, with hot water or with dilute acid or alkali or a detergent. Finally, fresh water is sucked through the entire apparatus for some time and allowed to discharge through the water jet pumps.

SUMMARY.

The construction and operation of a laboratory circulating evaporator which is capable of evaporating water at the rate of 12–14 l./hr. at 34–36° C. and 20–30 mm. Hg is described.

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ANTIBIOTICS FROM A STRAIN OF *B. SUBTILIS*: BACILIPIN A AND B AND BACILYSIN.

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THE production of a number of antibiotics has already been attributed to strains of *B. subtilis*, but since there is no universally accepted classification for this organism all workers may not have used the same species of aerobic spore-forming bacillus. One method of classification in present use is given by Gibson and Topping (1938) and Gibson (1945), and another by Smith, Gordon and Clarke (1946).

Two of these antibiotics, subtilin (Jansen and Hirshmann, 1944) and bacitracin (Johnson, Anker and Meleney, 1945) have been obtained in a relatively pure condition and shown to be polypeptides. The remainder have been less well defined and some may be identical. Among them, bacilysin (Gilliver, Abraham and Florey, 1946, unpublished; Florey, Chain, Heatley, Jennings, Sanders, Abraham and Florey, 1949), bacillin (Foster and Woodruff, 1946) and subtenolin (Hirschhorn, Bucca and Thayer, 1948) are probably peptides, while endosubtilysin (Olivier, de Saint-Rat, Bonét-Maury and Blanchon, 1945), subtilysine (Vallée, 1945*a*, 1945*b*) and eumycin (Johnson and Burdon, 1947) are probably organic acids.

The organism used in the present investigation (A14) was isolated by Miss M. Savage from a contaminated culture of *Myco. tuberculosis* brought from Chile by Dr. A. Arriagada. It was identified as a strain of *B. subtilis* by Miss K. Gilliver, using the method of Gibson and Topping (1938). On solid medium the growth of various bacteria, inoculated at right angles to a streak of the organism which had grown for 24 hours at 37° C., was inhibited to distances given in Column 1 of Table I.

This paper records the results of an investigation of the antibacterial products formed by the strain of *B. subtilis* A14 in aerated liquid media. Under the conditions used the organism produced at least three kinds of antibiotic.

MEDIUM AND CONDITIONS OF CULTURE OF THE ORGANISM.

The organism was grown in the salts of Czapek-Dox medium with the addition of 0·5 per cent neutral corn steep liquor and 0·5 per cent glucose. The medium was made up with tap water.

Antibacterial activity was measured by the cylinder plate method (Heatley, 1944), using *Myco. phlei* and *Staph. aureus* (H strain N.C.T.C. No. 6571) as test organisms.

When the organism was grown in stationary shallow layers at 37° C., the culture fluid showed no activity against either test organism. Aeration of the culture medium favoured antibiotic production. The most convenient temperature for growing aerated cultures was 34° C. At this temperature maximum activity was produced after twelve-hour growth from a 2 to 3 per cent inoculum of a nine-hour aerated culture.

The amount and quality of aeration was found to control the extent to which the antibacterial range of the culture fluid reproduced that found in the streak test. When aeration rates were low, or the bubble-size coarse, the culture fluid showed activity against *Myco. phlei* only. As the rate and efficiency of aeration was increased, activity against *Staph. aureus*, corynebacteria and gram-negative organisms was also noted, and this increased until an optimum aeration was reached. The actual amounts of air required varied with the shape and size of the culture vessel and the type of diffuser. Using glass bottles 4 in. in diameter containing one litre of culture fluid and an aloxite aerator stone 2 cm. × 2 cm. × 5 cm. (Carborundum Co.) the threshold amount to produce activity detectable by the cylinder-plate method against organisms other than *Myco. phlei* was two volumes per volume of culture fluid per minute (i.e. 2 l. per minute in these vessels). The optimum was reached at ten volumes per volume per minute. These very high rates could be reduced to one volume per volume per minute, and still give maximum production, in twenty-litre vessels 12 in. in diameter, when diffusers covering nearly the whole of the bottom of the vessel were used. The changes in pH and the percentage of the final activity produced at hourly intervals is shown in Fig. 1. Spores began to appear at 14 hours and by 20 hours only a few vegetative organisms remained.

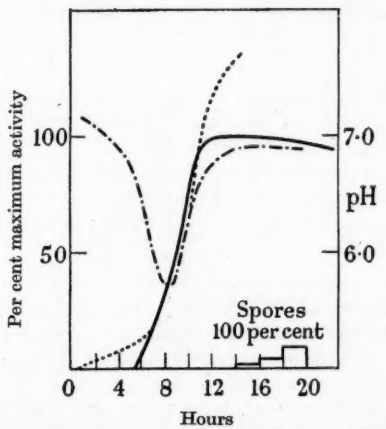

FIG. 1.—Activity and pH changes during aerated culture of *B. subtilis*. (A.14), 34° C. — Per cent maximum activity against *Staph. aureus* and *Myco. phlei* for both types of vessel described; ... per cent maximum activity *Myco. phlei* only; —·— pH, 1 l. vessels 10 vol. air/vol./min.; — pH, 20 l. vessels 1 vol. air/vol./min.

The antibacterial range of the culture fluid after 12 hours' incubation under conditions of optimum aeration is shown in Column 2, Table I.

TABLE I.

Organism.	(1) Length in mm. of inhibition of growth of secondary streak.	(2) Length in mm. of inhibition produced on a wheel-plate by aerated culture fluid.
Myco. phlei (Lab.)	14	15
Staph. aureus N.C.T.C. No. 6571	(11)	10*
C. diphtheriae var *gravis*	6	8
C. xerosis	4	8
Salm. enteritidis Gaertner	(10)	6
Salm. typhi	2	5
Streptococcus	0	0
Bact. coli	0	2
Ps. pyocyanea	0	0

( ) Partial inhibition only. * Some resistant colonies.

NATURE OF THE DIFFERENT ANTIBIOTICS.

The culture fluid contained three distinct groups of antibiotics.

Group I.

Two acidic substances which were readily extracted into organic solvents, other than petroleum ether, at pH 5·0, and which accounted for 75 per cent of the activity against *Myco. phlei*. The names bacilipin A and bacilipin B have been given to these substances.

Group II.

A stable peptide which remained in the culture fluid after extraction of the bacilipins. This was similar to, and was possibly identical with, an antibiotic mentioned by Abraham, Callow and Gilliver (1946) which has been called bacilysin (Gilliver, Abraham and Florey, 1946, unpublished). Bacilysin accounts for almost all of the activity of the culture fluid against *Staph. aureus*, and the coryne-bacteria, and for 25 per cent of the activity against *Myco. phlei*.

Group III.

An unstable substance (or substances) responsible for the activity of the culture fluid against gram negative organisms. Its activity in the harvested culture fluid was considerably reduced after 24 hours at 4° C., but it was more stable after heating the fluid at 70° C. for ten minutes. Possibly an enzyme system produced by the organism was responsible for its destruction. This group has so far been little investigated.

Bacilipin A and B.

Assay.

The cylinder-plate method of assay was used throughout the purification. *Staph. aureus* grown on heart-agar was used as a test organism. Although the

bacilipins only produce zones of partial inhibition when tested against *Staph. aureus*, this organism was found to give a more reliable assay than *Myco. phlei*.

Purification.

Extraction from culture fluid.—Bacilipins A and B are extracted quantitatively from the culture fluid, at pH 5·0, into a quarter of a volume of amyl acetate. Since back extraction into water was not quantitative and distillation produced considerable inactivation, the activity in the amyl acetate was concentrated on alumina by percolation through four columns in series. In this way the active material from one litre of amyl acetate concentrate could be adsorbed on 10 ml. of Brockmann alumina pH 9·0. An eluting mixture of one-fortieth of a volume of M/2 potassium phosphate just saturated with acetone was then run through the columns in series. The elution of the activity was not found to be correlated with any coloured bands. The combined eluates contained from 30 to 50 per cent of the activity originally in the amyl acetate; the remainder of the activity left on the columns tailed too much to warrant efficient recovery. The bacilipins were then extracted into ether, which was concentrated to a convenient volume *in vacuo* and further purified by the counter-current distribution method (Craig, Golumbic, Mighton and Titus, 1945). Early counter-current studies revealed the presence of two active substances, A and B (Fig. 2).

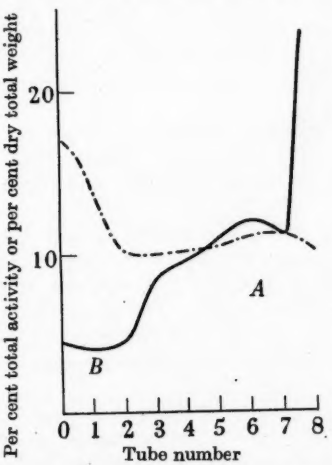

FIG. 2.—Counter-current distribution pattern of a mixture of bacilipins A and B. pH 6·8;
K (mixture) $\frac{\text{(Ether)}}{\text{(M/2 Buffer)}} = 1{\cdot}0$ approx;
Buffer mobile.
—·—, per cent total activity; —, per cent dry weight.

Separation of bacilipin A and B.—Titration curves for the transfer of the separated bacilipin A and B from ethereal to aqueous solution (Fig. 3) showed subsequently that A and B could be separated by fractional partition between ether and buffer solutions (Table II). This enabled the initial counter-current distribution to be dispensed with.

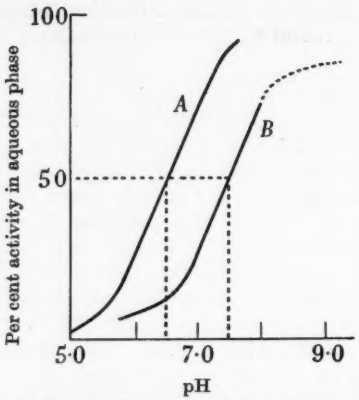

FIG. 3.—Distribution curves for bacilipins A and B between ether and M/2 potassium phosphate.

Counter-current distribution.—The separated fractions were each purified further by successive ten or twelve counter-current distributions in separating funnels. Ether was used as the static and M/2 potassium phosphate buffer as the mobile phase. The partition coefficients were approximately one when the buffer solution was at pH 6·5 for A and at 7·8 for B. Very stable emulsions were produced with this system unless the concentration of the crude substance was kept below 4 mg./ml.

The necessity of using dilute solutions of the active substances made the use of Craig's stainless steel apparatus (Craig, 1944) quite impracticable for work on a preparative scale, thus rendering the procedure more laborious and less efficient.

At the end of each run all the material in each tube was transferred to the ether layers, which were analysed to give the percentage of the total dry weight and total activity present in each. The method of selecting "strategic fractions" (Craig, 1944) was then used. The strategic fractions were pooled and further runs carried out, until some of the material behaved as though it was homogeneous, in that the partition coefficient remained constant over a number of tubes (Williamson and Craig, 1947).

Fig. 4 shows the first, second and third runs on "B" and Fig. 5 the first and fourth runs for "A." From two hundred titres of culture fluid 110 mg. of A and 60 mg. of B, in ethereal solutions, were shown to be practically homogeneous within the limits of resolution of the method.

Chemical properties.

Attempted isolation in the solid state.—On drying solutions of the free acids A and B in organic solvents, or solutions of their salts in water, an irreversible reaction occurred, even at low temperatures. From 50 to 70 per cent of the activity was lost initially and the loss increased further on standing. The loss of activity was accompanied by a decrease in the amount of hydrogen absorbed on catalytic hydrogenation and by a sharp decrease in solubility in organic solvents.

TABLE II.—*Separation of Bacilipin A and B.*

Crude ether concentrate, 2 l.

Extracted with :
(1) 750 ml. } M/2 potassium
(2) 500 ,, } phosphate buffer
(3) 250 ,, } pH 7·0.

Ether layer, 2 l. *enriched in bacilipin B.*

Extracted with :
(1) 750 ml. } M/2 potassium
(2) 500 ,, } phosphate buffer
*(3) 250 ,, } pH 9·0.†

Ether layer discarded.

Combined buffer solutions, 1.5 l. *enriched in bacilipin A.*

Combined buffer layers 1.5 l. *mixture of A and B.*

(*a*) pH adjusted to 7·65
(*b*) Extracted with :
(1) 750 ml. } Ether
(2) 500 ,, }
(3) 250 ,, }

Ether layer, 1.5 l. *almost free from bacilipin A.*

Buffer solution, 1.5 l, *mixture of A and B.*

Whole process repeated.

* Ether layer evaporated to 250 ml. for this extraction.
† Boric acid interferes when borate buffer is used.

Elementary analysis.—Elementary analyses of the lyophil-dried barium salts (Weiler and Strauss, Oxford) gave the following results :

A : C, 42·6 ; H, 6·3 ; N, 2·5 ; Ba, 24·6.

B : C, 52·45 ; H, 6·75 ; N, 2·09 ; Ba, 21·6.

Sulphur and phosphorous were absent.

Since these values are for the dried substances they may differ significantly from those for the fully active barium salts. Lack of material prevented an attempt to deduce the elementary composition of the active substances from analyses carried out after hydrogenation.

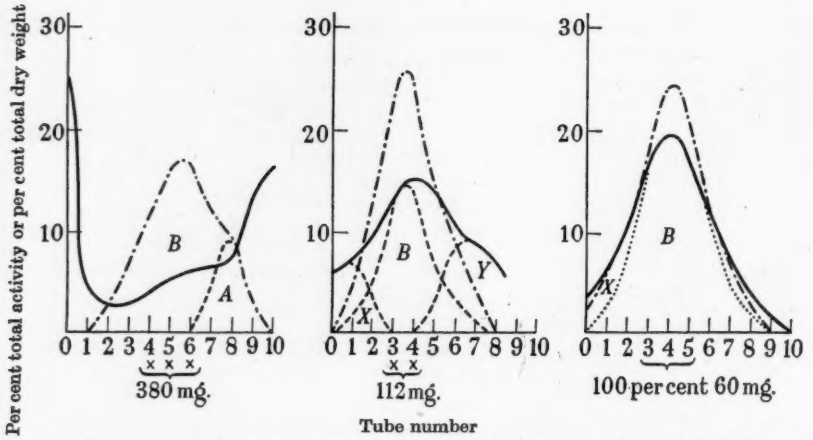

FIG. 4.—Three successive ten tube counter-current distributions on 2·5 g. crude bacilipin B, showing strategic fractions selected. pH 7·9; $K = \frac{(\text{Ether})}{(\text{M}/2\ \text{Buffer})} = 1{\cdot}4$; the aqueous phase was mobile.
—·—, per cent total activity; — per cent dry weight; ..., theoretical curve K = 1·4; — — —, approximate theoretical interpretation; xx, strategic fractions selected.

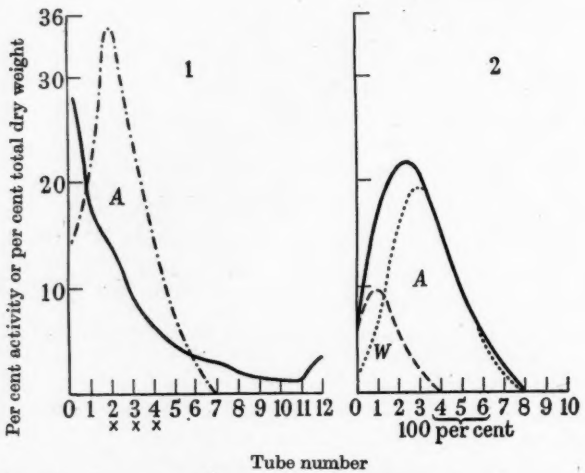

FIG. 5.—(1) Initial twelve tube distribution on crude bacilipin A.
pH 6·0; $K = \frac{(\text{Ether})}{(\text{M}\ \text{Buffer})} = 3{\cdot}3$ approx.
(2) Fourth run; pH 6·5; $K = \frac{(\text{Ether})}{(\text{M}/2\ \text{Buffer})} = 1{\cdot}7$; derived curve W is almost that for a homogeneous substance K = 6·7.
—·—, per cent total activity; —, per cent dry weight; ..., theoretical curve K = 1·7; — — —, (per cent total dry weight, minus theoretical curve A); xxx, strategic fraction selected.

The equivalent weight of the dry barium salt of A was 278 and that of B was 317·5. Since the ratio Ba/N was one this indicated that the minimum molecular weight of A was 556 and that of B was 635.

Solubility and general properties.—The free acids A and B were readily soluble in amyl acetate, benzene, chloroform and ether, but insoluble in petroleum ether. They had a very limited solubility in water, being precipitated as unstable oils on adjusting a neutral aqueous solution to pH 3·0. The sodium and barium salts were soluble in water and also in absolute alcohol.

Both A and B gave a negative Molish reaction, formed no precipitate with 2:4-dinitrophenylhydrazine and did not reduce ammoniacal silver nitrate.

Both substances readily absorbed bromine in chloroform solution. The bromo compounds were dark viscous oils and were biologically inactive.

Acid base properties.—Titrations made with a glass electrode showed that A was a stronger acid than B (Fig. 6). The titration curves showed no indication of the presence of a basic group.

After drying over phosphorous pentoxide the equivalent weight of A, found by titration to pH 7·6, was nearly double that of the undried substance. The original value of the equivalent weight was obtained, however, when further alkali was added and the solution was warmed. This suggested that lactonization had taken place.

Hydrogenation.—Hydrogenation of A and B in a Warburg manometric apparatus with an Adams or palladium/barium sulphate catalyst showed that the degree of unsaturation of the substances was similar but decreased after drying from aqueous solutions. Thus hydrogenation of the barium salt of A in aqueous solution gave the following figures for hydrogen uptake :

Before drying : 180 c.mm./mg.

After drying : 68 c.mm./mg.

The dry barium salt had lost 75 per cent of the activity shown by the solution. The difference in both hydrogen uptake and activity was even greater after the solid had been kept for a few days.

An oil was obtained when a solution of the hydrogenated sodium salt of A (undried) was acidified to pH 2·0. This crystallized in sheaves of needles m.p. 78° to 76° C. when the turbid solution was left in contact with petrol ether overnight. Extraction of the supernatant with ether yielded an oil mixed with some crystals of m.p. 95° to 100° C. The amounts of crystalline material available were insufficient for further investigation. Only oily material was obtained when hydrogenated B was treated in a similar manner.

Stability in solution.—In amyl acetate 25 per cent of the activity of a dilute solution of the free bacilipins A and B was lost after heating for one hour at 70° C.

Aqueous solutions of salts of the bacilipins were stable within the range pH 2·0—pH 10·0 at 16° C. for four hours but were rapidly destroyed at 100° C. At pH 13 inactivation of A and B occurred at room temperature. Subsequent electrometric titration indicated that the inactivation was accompanied by the liberation of a basic group and two new acid groups (Fig. 6). The products of inactivation did not give a ninhydrin reaction.

The activity of bacilipin A and B was not reduced by incubation with 50 per cent serum or with trypsin at pH 8.

Acid hydrolysis.—When 2 mg. of the barium salts of both A and B were heated with 6N hydrochloric acid in a sealed tube at 100° C. overnight, much "humin" was produced by B but considerably less by A. Extraction of the hydrolysates with ether and evaporation of the extract yielded an oil from A and crystals m.p. 105° C. from B.

The ether-insoluble material resulting from the hydrolysis of both A and B gave a positive ninhydrin reaction. The colour yield from A was about half, and from B a quarter, of that given by a standard solution of glycine containing an equivalent amount of nitrogen.

Paper strip chromatograms (Consden, Gordon and Martin, 1944), using phenol-5N hydrochloric acid, were run with 5 μl. (= 5 γN) of a solution of the hydrolysates of A and B but no spots were revealed on treatment with ninhydrin. After hydrolysis about one-fifth of the total nitrogen was present as volatile base, and this was probably responsible for the ninhydrin reaction.

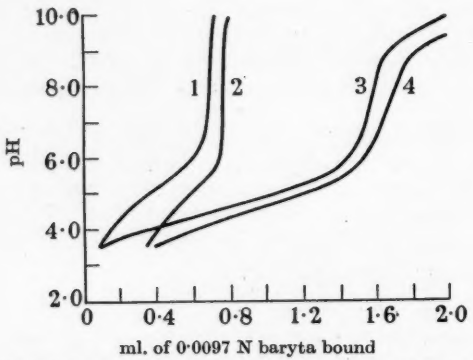

FIG. 6.—Electrometric titrations on 2·04 mg. bacilipin A and B before and after inactivation at pH 13·0. Curve (1), active bacilipin B; Curve (3), inactive bacilipin B; Curve (2), active bacilipin A; Curve (4), inactive bacilipin A.

Discussion.

Bacilipins A and B appear to be similar and to be chemically related. The limited chemical information that could be obtained from the small amounts of material available indicates that the bacilipins are nitrogenous unsaturated aliphatic dibasic acids. Since two acid groups and a basic group are liberated by M/10 sodium hydroxide the substances probably contain an amide type of linkage. The base does not give a ninhydrin reaction and is not volatile. The other acid group liberated may arise from the hydrolysis of an ester linkage or the opening of a lactone ring.

However, the results of a single elementary analysis of the dried barium salts of both bacilipin A and B appeared to reveal a degree of saturation which does not allow all these groupings to be fitted into the molecule and also account for the observed hydrogen uptake of the active substances on catalytic hydrogenation. It is possible that the barium salts are still hydrated after drying and that the inactivation which occurs on drying involves oxidation across the double bonds.

Since the antibacterial properties of the compounds did not justify the considerable amount of work which would have been required to obtain larger amounts of the pure substances, further investigations on bacilipin A and B have been discontinued.

Bacilysin.

Partial purification.

Bacilysin was assayed by the cylinder-plate method (Heatley, 1944) throughout the purification. *Staph. aureus* grown on heart agar was used as the test organism.

The culture fluid was acidified to pH 3·0 and clarified in a Sharples centrifuge. The pH was then adjusted to 2·0 and 1·5 per cent Farnell phosphoric-activated carbon was added. The mixture was agitated for twenty minutes and then filtered. The bacilysin was eluted by two extractions with a quarter volume of a mixture of four volumes of ethanol and one of M/2 potassium phosphate pH 7·0. This amount of phosphate buffer is just sufficient to saturate the alcohol phase and leave a small volume, about 50 ml. per litre, of the aqueous phase. Only the alcohol phase appears in the eluates. Overall recovery under favourable conditions has been 70 per cent.

The alcoholic eluate was percolated rapidly through a column of Brockman alumina pH 5·0 until the column was nearly saturated (300 ml. per 10 ml. alumina). Bacilysin was readily eluted from the alumina by raising the water content of the solvent. Elution with 25 per cent ethanol was found to give material of the highest activity. There appeared to be no correlation between bands visible under U.V. light and the active material. A standardized ninhydrin colour reaction carried out on aliquots of the effluent fractions, showed that inactive peptides or amino acids continued to be eluted after the bacilysin had been removed from the column. Bacilysin was obtained as an amorphous white powder when the eluates were distilled to a small volume *in vacuo* and the aqueous residue lyophil-dried. Material obtained in this way from the most active " cut " from the columns was used in some preliminary chemical investigations.

Chemical properties.

The preparation of bacilysin contained nitrogen and sulphur. It was practically insoluble in all dry organic solvents except methanol. It was readily soluble in water, and its solubility was not appreciably reduced by saturation with ammonium sulphate. An aqueous solution formed no precipitate with picric acid, phosphotungstic acid, trichloroacetic acid or basic lead acetate. It gave positive ninhydrin and Pauly diazo reactions. The Molish test was negative.

Stability.

Bacilysin was stable for 4 hours at 20° C. between pH 1·4 and 12·0. It was not destroyed when heated to 100° C. for fifteen minutes at pH 7·0 to 9·4. However, when heated for ten minutes at pH 1·0 over 90 per cent of the activity was lost.

Incubation with crystalline trypsin and with tissue enzymes at pH 8·0 rapidly inactivated bacilysin. Treatment with hydrogen sulphide reduced the activity by half.

Hydrolysis and paper chromatography.

Paper chromatograms after hydrolysis with 6 N HCl at 100° C. showed that the material contained alanine, tyrosine, leucine and phenylalanine.

The evidence at present available indicates that bacilysin is a neutral peptide.

ANTIBACTERIAL PROPERTIES OF BACILIPIN A AND B AND BACILYSIN.

Bacilipins A and B.

Cylinder-plate test.—Assay by the cylinder-plate method using *Staph. aureus* and *Myco. phlei* as test organisms gave zones of inhibition 20 to 25 mm. in diameter at the following dilutions.

	Staph. aureus.	*Myco. phlei.*
A	1/10,000	1/100,000
B	1/80,000	1/800,000

The plates were seeded with a 12-hour broth culture of *Myco. phlei*, and with a 12-hour culture diluted 1:100 of *Staph. aureus*. They were read after 12-hour incubation.

The inhibition was only partial with *Staph. aureus* but remained constant on further incubation. With *Myco. phlei* the zones of inhibition were clear but were much reduced after 48 hours' incubation.

Wheel-plate test.—When tested by the wheel-plate method at 1/1000 concentration against various bacteria, the lengths of inhibition shown after 15-hour growth by the susceptible organisms were as follows :

	Staph. aureus.	*Myco. phlei.*	*B. anthracis.*
A	(7 mm.)	15 mm.	5 mm.
B	(15 ,,)	20 ,,	12 ,,

( ) partial inhibition.

Bact. coli, *Salm. enteritidis* Gaertner, *C. xerosis*, *Strep. pyogenes* strain, Kruger, *C. diphtheriae* var. *gravis* and *Ps. pyocyanea* were not inhibited.

Serial dilution test.—Serial dilutions using *Myco. phlei* as test organism made with an impure mixture of A and B of unknown relative composition gave an endpoint of 1/320,000 when read at 15 hours' growth, but this decreased to zero after 48 hours. Pure B was later shown to be about twenty times more active by the cylinder-plate method than the sample used in this test.

Partially-purified bacilysin.

Wheel-plate test.—When tested by the wheel-plate method at a concentration of 1/1000, bacilysin inhibited the growth of various organisms to the following distances from the cylinder :

Bact. coli.	*Staph. aureus.*	*C. xerosis.*	*Myco. phlei.*
(5 mm.).	10 mm.	10 mm.	(5 mm.).

The inhibition of *Bact. coli* and *Myco. phlei* was only partial and decreased after 38 hours' incubation. A few resistant colonies were found within the zone of inhibition against *Staph. aureus* (Abraham, Callow and Gilliver, 1946). *B. anthracis* and *Strep. pyogenes* were not inhibited.

Combined action of bacilysin and the bacilipins.

Cylinder-plate test.—When the culture fluid was tested by the cylinder-plate method against *Staph. aureus* or *Myco. phlei*, the zones of inhibition were clear, and remained constant in diameter if the period of incubation was extended from 18 to 48 hours. In contrast, the pure bacilipins did not produce a permanent zone of inhibition when tested against *Myco. phlei*, the zones gradually diminishing in size through growth at the circumference, and when bacilysin was tested against *Staph. aureus*, resistant colonies were found within the zone of inhibition. A reconstructed mixture having similar concentrations of the antibiotics to those in the culture fluid gave the same type of inhibition as that of the culture fluid itself (Fig. 7).

Serial dilution method.—Serial dilution tests were made with bacilysin alone and with a mixture of bacilysin and bacilipin A (10:1), using *Staph. aureus* as the test organism. In both cases visible growth was inhibited after 16 hours incubation when the concentration of bacilysin was 1 in 320,000. On further incubation all the tubes containing bacilysin alone eventually showed the same turbidity as the control, but in the tubes containing the mixture of antibiotics further growth was considerably retarded and inhibition was still complete at 1 in 20,000 after 4 days.

Conclusion.

It appears that the bacilipins are bacteriostatic towards *Myco. phlei*, for while they initially inhibit growth at high dilutions multiplication finally occurs and the organisms in the resulting culture have no increased resistance. Bacilysin delays visible growth of *Staph. aureus* in high dilutions, but in this case there is also eventual multiplication. The effect of bacilysin is due to a partial bactericidal action followed by the growth of resistant surviving cells (Abraham, Callow and Gilliver, 1946).

Bacilysin and the bacilipins combined are considerably more effective in suppressing bacterial growth than is either alone. This accounts for the fact that the crude culture fluid produces clearer or more permanent zones of inhibition than the purified antibiotics when tested by the cylinder-plate method.

RELATIONSHIP OF THE BACILIPINS AND BACILYSIN TO OTHER ANTIBIOTICS FROM *B. subtilis*.

Bacilipins.—Other organic acids so far obtained from *B. subtilis* appear to be distinct from the bacilipins. Thus, endosubtilysin (Olivier, de Saint-Rat, Bonét-Maury and Blanchon, 1945) is insoluble in acetone. Subtilysine (Vallée, 1945*a*, 1945*b*) is insoluble in 10 per cent calcium chloride and eumycin (Johnson and Burdon, 1946) is insoluble in ether and amyl acetate.

The active principle "M. and R." (Miller and Rowley, 1948) obtained from a *B. mesentericus* shows similarities to the bacilipins in its general chemical pro-

perties. However, the most purified sample of M. and R. contains at least twice as much nitrogen as the bacilipins and is active against a much wider range of organisms. It would appear that strains of *B. subtilis* produce a number of organic acids having antibacterial properties, in addition to several biologically active peptides.

Bacilysin.—Among the antibacterial peptides produced by strains of *B. subtilis* are bacitracin, subtenolin, subtilin and bacillin. The fact that bacitracin and subtenolin form a relatively insoluble picrate distinguishes them from bacilysin and also from subtilin and bacillin. Of the latter three substances, subtilin, unlike bacilysin, is readily salted out from aqueous solution and contains at least twelve different amino acids, while bacillin is equally active against gram-positive and negative organisms, when tested in nutrient agar. Bacilysin, therefore, appears to differ from other antibacterial peptides hitherto described.

SUMMARY.

A strain of *B. subtilis* A14 produces four different antibiotics when grown in submerged aerated culture in a modified Czapek-Dox medium. Two of the antibiotics can be extracted by organic solvents and have been named bacilipin A and bacilipin B. A third antibiotic is insoluble in organic solvents and is called bacilysin. The fourth substance differs from the other three in that it is active against *Bact. coli* and is rapidly destroyed in the harvested culture medium.

Bacilipin A and bacilipin B are unsaturated aliphatic nitrogenous acids and have similar properties. They are stable in solution at room temperature but have only been isolated in the solid state with loss of activity.

Bacilysin is a water-soluble neutral peptide which appears to be of relatively simple composition. The most active preparation yields alanine, tyrosine, phenylalanine and leucine on hydrolysis. Bacilysin is inactivated by trypsin and tissue enzymes.

The bacilipins are active mainly against *Myco. phlei* and their action appears to be bacteriostatic. Bacilysin is active against *Staph. aureus*, *C. xerosis*, *Myco. phlei* and to a lesser extent *Bact. coli*. Its action is mainly bactericidal. However, when tested against *Staph. aureus* some organisms survive and these are found to be resistant to bacilysin after subculturing. Mixtures of the bacilipins and bacilysin show a combined action, which is more effective in suppressing bacterial growth than is either component when tested alone.

I am indebted to Dr. E. P. Abraham for guidance and advice in the course of this work, to Dr. N. G. Heatley for helpful suggestions, to Miss M. Lancaster and Miss M. Bond for the tests of antibacterial activity carried out under the supervision of Dr. M. A. Jennings and Sir Percival Hartley, to Miss K. Gilliver and Mr. J. Kent for help in producing batches of culture fluid, and to Miss J. Moss and Miss A. Pill for technical assistance.

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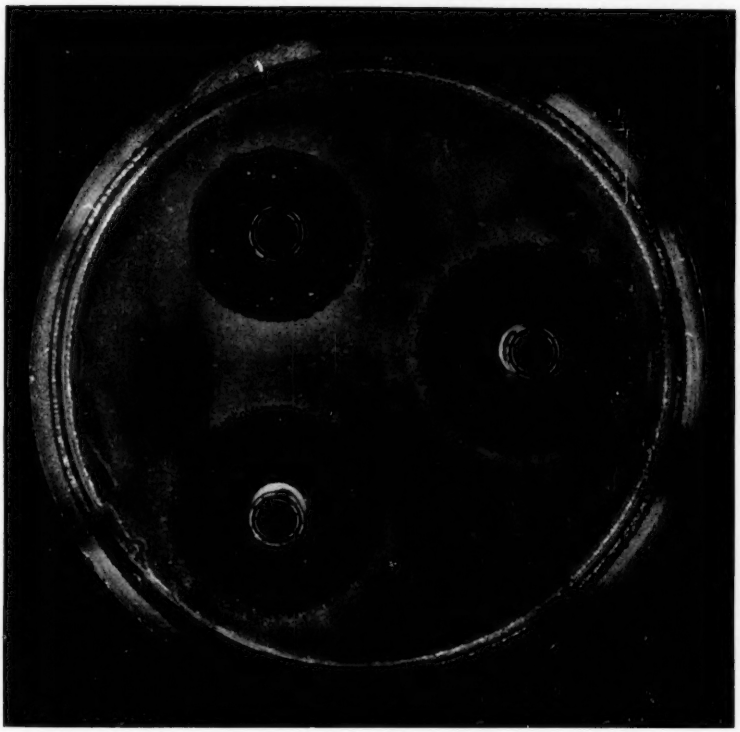

FIG. 7.—Combined action of bacilysin and bacilipin B against *Staph. aureus*. Top left zone, bacilysin 1/4000 showing resistant colonies; bottom left, bacilipin B 1/10,000 showing partial inhibition only; right, a mixture containing bacilysin 1/4000 and bacilipin B 1/10,000 showing a zone of complete inhibition.

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THE FIXATION OF DIPHTHERIA TOXIN TO SKIN TISSUE WITH SPECIAL REFERENCE TO THE ACTION OF CIRCULATING ANTITOXIN.

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FROM a study of the intoxication of rabbit tissues by the α-toxin of *Cl. welchii*, Miles and Miles (1943) concluded that the inefficacy of intravenous antitoxin given a few hours after the local injection of toxin was due primarily to the pressure of intracellular exudate in the toxin lesion, which opposed the exudation of antitoxin-containing plasma. The conclusion is applicable to all toxins that rapidly and greatly increase the permeability of the blood capillary endothelium. However, late-administered antitoxin is also ineffective with toxins like that of *C. diphtheriae*, which does not greatly change the capillary permeability, at least during the first few hours of intoxication.

The inaccessibility of diphtheria toxin to circulating antitoxin was measured in guinea-pigs by Glenny and Hopkins (1925). Intradermal toxin was partly neutralized by intravenous antitoxin given 1 to 2 hours after the toxin. Friedemann and his colleagues (Friedemann and Zuger, 1939; Friedemann, Zuger and Hollander, 1939*a*, 1939*b*) gave antitoxin before and after intradermal toxin in the rabbit. The antitoxin was equally effective whether it was given 2 minutes or 3 hours before the toxin, from which they concluded that a certain concentration of antibody was established in extravascular tissues immediately after

injection, and this determined the neutralization of intradermal toxin at the time of its injection into the skin. These conclusions were in accord with the fact that when antitoxin was given *after* the toxin, its efficacy was greatly diminished, even when the toxin lesions were only a few minutes old. They also measured the amount of antitoxin neutralizing toxin *in vitro* (the *direct* neutralizing quantity, symbolized by *Ad*) and *in vivo* after intravenous injection (the *indirect* neutralizing quantity, symbolized by *Ai*) and found the ratio *Ai : Ad* to be 22,500 : 1. Allowing for the plasma volume of the rabbit and the volume of toxin injected, and assuming that tissues and antitoxin did not compete for the toxin, they deduced a ratio of 22·5 : 1 for the relative concentration of antitoxin in the circulation and the tissues.

The concepts of a rapidly established concentration of antibody in the tissues, fully effective (i.e. *definitive*) in the sense that when toxin is injected, no antibody subsequently passing into the tissues from the blood adds to the neutralizing effect; and of a rapid and irreversible fixation of toxin to the tissues in the absence of antibody, have a direct bearing on passive antibody therapy; and were accordingly re-examined.

MATERIALS AND METHODS.

One batch of diphtheria toxin and one of antitoxin were used throughout. The toxin was prepared by Dr. L. Holt on a semi-defined medium; it contained 0·1 per cent total N and 78 Lf per ml.; at Lf 52 its Kf was 10. It was preserved at 2° C. under toluene. The antitoxin was a commercial preparation of proteolytically refined horse serum containing 4000 international units (u.) per ml.; standard stock dilutions were made in 50 per cent glycerol saline and held at 2° C. Both reagents were diluted for use in saline, usually in a geometric series, using Grade A pipettes, burettes and volumetric flasks. The cumulative errors of serial dilution were avoided as far as possible by adding "master" solutions to various volumes of saline in large vessels. Thus, for a 2-fold series the volumes in ml. respectively of toxin master-solution and saline were 3 + 3; 2 + 4; 1 + 7; 1 + 15; 1 + 31; 1 + 63; 1 + 127.

Except when otherwise stated, laboratory stock albino guinea-pigs of 250 to 400 g. weight were used; for any one test the weight range was usually within 80 g. The animals were clipped, depilated by a barium sulphide paste 18 hours before the tests, and all animals with the slightest evidence of a depilation "burn" were rejected. A minimum of three animals was used for each titration. Intradermal injections of 0·1 ml. were made with needles of No. 26 gauge.

The intensity and diameter of areas of inflammation were measured after 24 hours, correct to the nearest half-millimetre. The diameter of all round lesions was recorded, and the major and minor diameters (D and d) of the remaining slightly elliptical lesions reduced to a single measure, $\sqrt{Dd}$. The responses to series of intradermal doses of toxin were subjected, when necessary, to regression analysis (Fisher, 1947).

EXPERIMENTAL.

The dose-response to intradermal diphtheria toxin.

Current methods of titration usually depend on the determination of an endpoint, which is embodied in the conception of the minimal skin-reacting dose.

In practice this appears to be the amount of toxin producing an inflammatory lesion between 8 and 15 mm. in diameter. For example, Jensen's (1933) " DRM " gave a lesion 8 to 10 mm. in diameter. Amounts of toxin producing lesions larger than 30 to 35 mm. are impracticable because of the ultimate, perhaps fatal, general intoxication following the doses necessary to produce lesions of this size ; and lesions smaller than 6 mm. may be confused with the faint but definite area of inflammation, 3 to 4·5 mm. in diameter, induced by injecting 0·1 ml. of 0·85 per cent saline. Between these two limits there is a practicable range of doses and, since estimates of toxicity depending on the responses to more than one dose are preferable to those depending on an end-point, the dose-response to diphtheria toxin in the skin was investigated within this range.

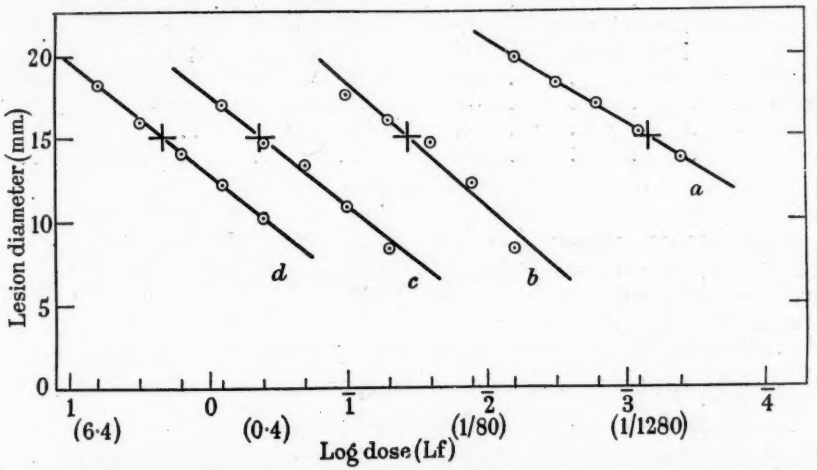

FIG. 1.—Indirect neutralization of intradermal diphtheria toxin by antitoxin. Linearity and parallelism of the dose-response curves.

(*a*) Antitoxin *nil*.
(*b*) „ 60 u. per kg. guinea-pig body weight.
(*c*) „ 600 u. „ „ „ „ „
(*d*) „ 6000 u. „ „ „ „ „

The dose-response lines were fitted from the means of six lesion-diameters in three guinea-pigs.

The curve obtained by plotting diameter (d) of the 24-hour inflammatory area against the logarithm of the dose (x) proved to be linear (Fig. 1, 2*a*, 4) ; that is, $d = b \log x$, where b is the slope of the regression line. The slope was remarkably reproducible ; for example, in 13 assays picked at random from a series made over 7 months, the slopes were : — 6·75, — 7·59, — 5·97, — 5·94, — 7·03, — 5·21, — 5·91, — 6·78, — 5·81, — 7·59, — 6·57, — 6·54, — 5·23. The relative constancy of slope may depend on the age of the lesion, the batch of toxin and the animal used. Jensen (1933) measured lesion-diameters in rabbits at 36 hours. His Table 7 records titrations of a toxin in 15 rabbits ; the diameters are given correct to the nearest 2 mm. (i.e. 8, 10, 14, etc.). By regression analysis of Jensen's figures, the dose-response proved to be linear in 12 of 15 rabbits, and in these animals the slope varied between 10·6 and 27·5.

It was important to prove that this convenient linear dosage-response relation held for all parts of the guinea-pig skin used in the titrations. For a given dose of toxin in 0·1 ml., the lesions in the ventral half of the body skin are bigger than

TABLE I.—*The Analysis of Variance of the Diameter of the Toxin Lesions in the Skin of Six Guinea-pigs in Response to Four Doses of Diphtheria Toxin, Randomized in a Four-fold Latin Square.*

Source of variation.	DF.	Mean square error.	F.	P.
Between AP* rows	3	7·1485	4·71	0·001–0·01
,, T* rows	3	2·2596	1·49	>0·05
,, doses	3	347·5859	228·9	<0·001
Linearity	1	1028·1380	677·2	<0·001
Departures	2	7·3099	4·81	0·01–0·05
Between guinea-pigs	5	1·9422	1·28	>0·05
Interactions	45	1·8861	1·24	>0·05
Residual	36	1·5182	..	..
Total	95	..	..	..

* See p. 322.

in the dorsal half. Titrations were therefore confined to an area on the two sides of the spine, bounded laterally in the sitting animal by the horizontal lines midway between the spine and the anterior belly wall, anteriorly by the vertical line through the elbow-joint, and posteriorly by the vertical line through the knee-joint. Within this approximately square area on the dorsal aspect of the animal it was possible to make sixteen equally spaced injections, 2 rows of 4 on each side of the spine.

The linearity of the dose-response and its independence of the site of injection within the chosen area were established by titrating four doses of toxin distributed randomly among the 16 available sites in Latin squares. Three tests were made, each in 6 to 8 animals. Table I records the analysis of variance from one test, typical of the three. Doses of 1/400, 1/1600, 1/6400 and 1/25,600 Lf were given to six guinea-pigs, so that each dose was tested 24 times. From column F (the mean square errors in terms of the residual mean square error 1·5182) it is clear that the variation due to position in the injection area, indicated by the variations in the response in the four antero-posterior (AP) rows of lesions is small compared with the difference between doses, and that in the four transverse (T) rows it is negligible. In two similar tests the variation between both T and AP rows was negligible. For practical purposes the skin of this area may be considered homogeneous with respect to toxin. In most subsequent tests, therefore, no formal randomization of doses among the available sites in all the animals in each test batch was attempted, though the pattern of injections was varied when possible. In this example the variation between animals is small ; in other tests it was larger, and sometimes significant ; but in all the cases analysed each animal provided a substantially linear response and the skin was uniformly susceptible to the toxin.

The value of F for linearity is satisfactorily significant, and for departures from linearity it is small. It follows that, by the simple transformation of dose

into log dose, the information given by the mean response to all the doses injected can be combined to obtain single estimate of toxicity. For this purpose a regression line is fitted and log x determined for an arbitrarily selected diameter of lesion. Values between 12·5 and 15 mm. were chosen, being near the mid-value of lesion-diameters usually obtained in the titrations. They are rather larger than the diameters produced by the orthodox " minimal reacting dose," but for two reasons they are more reliable measures of toxicity ; they summarize the response, not to one dose, but to a series of doses ; and they do not depend on personal judgement as to what a standard minimal reacting dose is. In this connection it may be noted that the lesion-diameter of a single dose is not a very sensitive indicator of variation in toxin concentration. The average slope is about -7 ; and since $d = -7 \log x$, the lesion diameter diminishes only by 2·1 mm. even for a 50 per cent decrease in concentration.

The dose-response to indirectly neutralized toxin.

The dose-response to toxin in animals with circulating antitoxin is also linear, and the slope is parallel to that for toxin alone, even in animals receiving very strong antitoxin. Each fitted slope in Fig. 1 summarizes six titrations, two per guinea-pig. Similar results were obtained in two repetitions of the test. The linearity and parallelism of the slopes are obvious ; and on analysis the four regression lines were homogeneous, the deviation of each from the common regression line being insignificant. Taking a 15 mm. lesion for the point of comparison, the relative toxicities are as follows : 60 u. antitoxin per kg. diminishes toxicity 51·4-fold ; 600 u., $11{\cdot}4 \times 51{\cdot}4$-fold ; and 6000 u., $5{\cdot}16 \times 11{\cdot}4 \times 51{\cdot}4 =$ 3,023-fold. The 5·16-fold decrease in toxicity for the 10-fold increase in antitoxin injected (600 to 6000 u.) compared with the 11·4-fold decrease for the step 60 to 600 u. suggests that with very strong intradermal toxin the constant proportionality between toxin and antitoxin, noted by Friedemann (1947) in the rabbit, does not hold (see p. 326).

At the lower end of the curves, where lesion-diameters are $<$6–8 mm., there was sometimes a departure from linearity, the curves dipping more steeply to the 3–5 mm. values. These values, covering the range of diameters of the mild inflammatory lesions produced by saline alone, must, for practical purposes, be considered as the base line of non-toxicity. Nevertheless, as in Fig. 5, I, with low concentrations of circulating antitoxin, the curves meet this base line at points whose distance apart is equal to the log decrease in antitoxin concentration. That is to say, with concentrations of toxin up to 1000 times that giving a 12·5 mm. lesion in the normal animal, the degree of indirect neutralization is proportional to the concentration of circulating antitoxin, whether the neutralization is measured by shift of the dose-reponse slope or, as Friedemann and his colleagues found, by shift of end-point. But with very strong toxin, simple proportionality does not hold, and antitoxin is less efficient.

The dose-response to directly neutralized toxin.

The picture is strikingly different for *in vitro* mixtures of toxin and antitoxin ; Fig. 2 is typical of the dose-responses to mixtures of a constant amount of antitoxin and graded amounts of toxin, held at 37° C. for 1 hour before injection. They differ sharply from those in Fig. 1 ; the upper part is approximately parallel

to that of toxin alone, but for lesion-diameters less than 17 to 18 mm., the curves dip steeply to the base-line at 3 to 5 mm. In the similarly steep portions of the curve that sometimes occurred in indirect neutralization (Fig. 5, I) the change of direction occurred only at lesion diameters of 6 to 8 mm., i.e. close to the lower end of the dose-response line.

As in Fig. 1 and 5, I, the ratio of these end-point values is equal to the ratio of antitoxin concentration used. From several titrations of this kind, the slope of this lower part of the curve was estimated as approximately — 35, a value

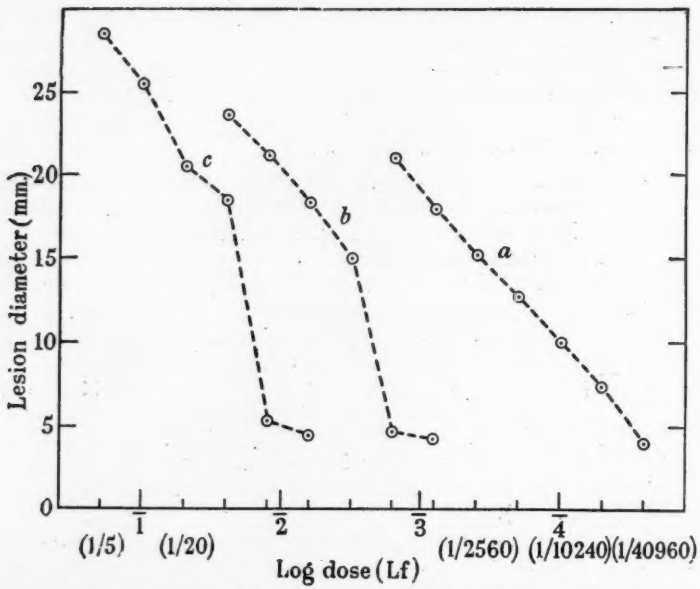

FIG. 2.—Direct neutralization of intradermal diphtheria toxin by antitoxin. The non-linearity of the dose-response curve, and absence of parallelism with the curve for toxin alone.

(*a*) Antitoxin *nil*.
(*b*) „ 0·0025 u.
(*c*) „ 0·02 u.

that differs significantly from the slope for toxin alone or for indirectly neutralized toxin, which ranged from — 5 to — 9. It is presumably the steepness of the slope in direct neutralization that permits accurate intradermal measurement of antitoxic potency by titration against a fixed amount of toxin, since an end-point can be estimated with fair precision.

The dose-response to toxin indirectly neutralized by late-administered antitoxin.

Returning to indirect neutralization, we find that variation in the period between injecting toxin and antitoxin has a notable effect which is readily measured in the same animal by injecting toxin at varying times before and after the intravenous injection of antitoxin. Comparison of the dose-response to

toxin indirectly neutralized by antitoxin given before and after toxin, established the following facts.

With small doses of toxin, requiring relatively little circulating antitoxin, there is no significant difference between the curves for antitoxin given 6 hours, 2 hours, and 3 minutes (— 3) before the toxin, and 3 minutes after (+ 3) the toxin. Antitoxin given even 60 minutes later (+ 60) has considerable neutralizing power (Fig. 3, typical of several tests). By regression analysis, the difference between the — 3 and + 3 curves was not significant, and between — 3 and

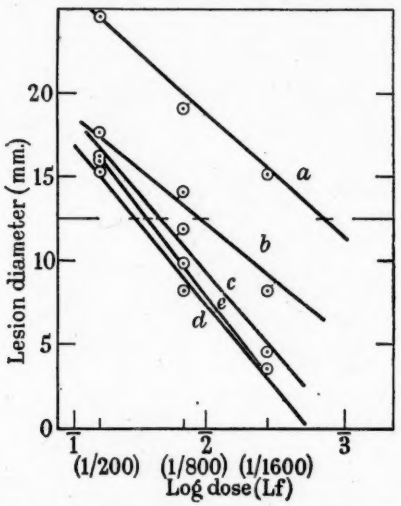

FIG. 3.—Indirect neutralization of intradermal diphtheria toxin by 1·5 u. antitoxin per kg. Similarity of neutralizing potency of antitoxin injected three minutes before and after the toxin.

(*a*) Toxin in animals without antitoxin.
(*b*) „ given 60 minutes before antitoxin.
(*c*) „ „ 30 „ „ „
(*d*) „ „ 3 „ „ „
(*e*) „ „ 3 „ after „

The dose-response lines were fitted from mean lesion-diameters in five guinea-pigs.

+ 30 only just so ; and, taking a lesion-diameter of 12·5 mm. for comparison, the potencies of the toxin in terms of its — 3 value, and the fiducial limits of error were :

Antitoxin.	Potency of toxin.	Limits (P = 0·95).
None	12·28	7·41–21·27
60 min. late	2·50	1·53–4·18
30 min. late	1·37	0·83–2·25
3 min. late	0·84	0·51–1·38

Thus, even when the antitoxin is given 60 minutes late, only 2·5/12·28 = one-fifth of the toxin is not neutralized.

The constant proportionality of toxin and antitoxin, already noted in Fig. 5, I, where the antitoxin was given at − 10 (i.e. 10 minutes before toxin), is disturbed when antitoxin is given late. In Fig. 5, II, with antitoxin given at + 10, the ratios of toxicity with the different antitoxin levels, as far as can be estimated, are much less than the expected value of 4.

In Friedemann and Zuger's (1939) Tables 3 and 4, the greatest effect of the late administration of antitoxin occurs with the strongest antitoxin. This is also evident in Fig. 4, where with 1 u. antitoxin per kg. the neutralization curves at − 12 and + 60 differ little, whereas the curve for 9 u. antitoxin per kg. has

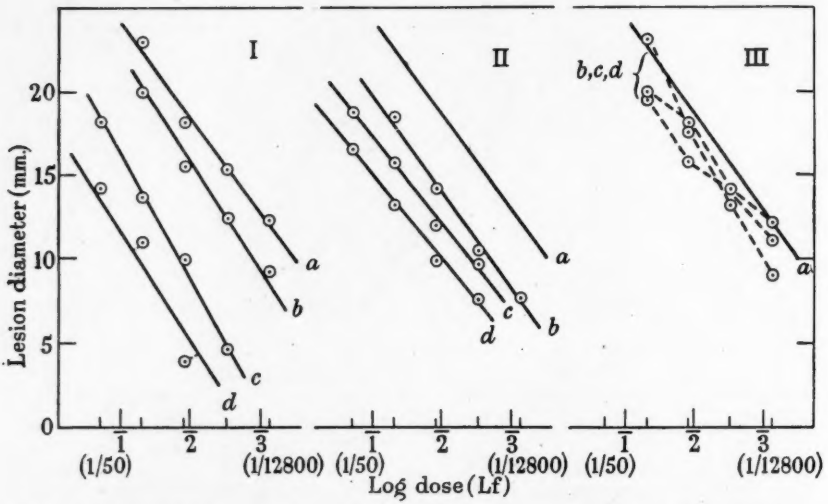

FIG. 4.—Indirect neutralization of intradermal diphtheria toxin by antitoxin. Increasing loss of neutralizing potency with increasing lapse of time between giving toxin and antitoxin.
I. Toxin given 12 minutes after antitoxin.
II. " " 60 " before "
III. " " 120 " " "
(*a*), (*b*), (*c*) and (*d*) respectively are the dose-response lines from guinea-pigs receiving 0, 1, 3 and 9 u. antitoxin per kg.

moved up considerably at + 60. The effect is presumably a consequence, not of the high concentration of antitoxin in the plasma, but of using the strong toxin, which in turn entails the use of the strong antitoxin. Even with pre-administered antitoxin, proportionality is disturbed when the toxin is very strong (Fig. 1*d* and *c*), and this disturbing effect of strong toxin appears to be exaggerated when the antitoxin is injected late. Fig. 4 also illustrates the fact that the indirect neutralizing power of antitoxin is small when the toxin lesions are two hours old.

These results offer little ground for supposing that a sharp distinction can be made between indirect neutralization by antitoxin given before and after toxin. At the critical point, the moment of injection of toxin, the change is gradual. The effect, however, is not symmetrical about this point, for whereas the diffe-

rence between antitoxin given 3 and 120 minutes early is negligible (see also Friedemann and Zuger, 1939), that between 3 and 120 minutes late is striking.

The Implication of the Dose-response to Diphtheria Toxin.

The results so far reported largely confirm those of Friedemann and his colleagues. There are, nevertheless, certain features of the dose-response curves for directly and indirectly neutralized toxin which suggest several objections to Friedemann's hypothesis.

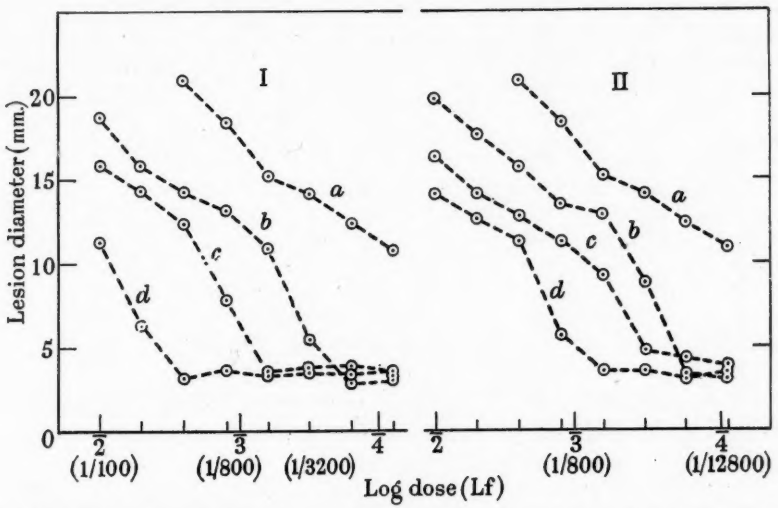

FIG. 5.—Indirect neutralization of intradermal diphtheria toxin by antitoxin. The effect of late administration of antitoxin on the constant proportionality of skin toxicity and blood concentration of antitoxin.

I. Toxin given 10 minutes after antitoxin.
II. " " 10 " before "

(*a*), (*b*), (*c*) and (*d*) are respectively the dose-response curves from guinea-pigs receiving 0, 1·5, 6 and 24 u. antitoxin per kg.

Direct neutralization.

The characteristic change of slope in the dose-response to mixtures of toxin and antitoxin may be simply explained. In Fig. 2*b* the lesion from 1/640 Lf is diminished to the size of that of 1/40960 Lf (i.e. a 64-fold drop) by 0·0025 units antitoxin, which therefore neutralizes 63 of these minimal doses (m.d.) of 1/40960 Lf. Since the antitoxin in these toxic mixtures may be assumed to be fully saturated with toxin, we may set down a series of doses of toxin in terms of this m.d., increasing 2-fold ; and if from each figure we subtract 63, equivalent to the neutralizing power of the antitoxin added, we have as follows :

Toxin (m.d.).	Antitoxin added.	Free toxin.	Ratio dose of free toxin to preceding dose.
16	63	0	..
34	63	0	..
64	63	1	∞
128	63	65	65·00
216	63	193	2·97
512	63	449	2·28
1024	63	961	2·18
2048	63	1985	2·07

Instead of a constant doubling of dose of free toxin, the effective doses (Column 3) increase rapidly at first, and then, as the ratios in Column 4 show, approximate more and more to the 2-fold increase. The response to such a series would correspond closely to that found (Fig. 2*b*), and, moreover, with stronger antitoxin, the length of the steeper part of the hypothetical dose-response curve would be increased, as happens in Fig. 2*c*.

The large difference between the slopes of the dose-response to toxin neutralized directly and indirectly (about -35 and -7) means that Friedemann's ratio $Ai : Ad$, will vary with the degree of inflammation chosen as end-point in the toxin titrations. In Fig. 2*a* the slope for toxin alone represents the slope for indirectly neutralized toxin, since (Fig. 1) the one is parallel to the other ; and a comparison of this slope with those for direct neutralization clearly shows how the non-parallelism will affect estimates of antitoxic efficiency at different lesion-diameters. Even if consistent results were obtained with a well-defined end-point due to weak toxin, the ratio would not be a valid basis for deducing a blood : tissue ratio of antitoxin in lesions due to stronger toxin.

Indirect neutralization.

Friedemann's hypothesis postulates the establishment, very soon after its injection, of a protective layer of antitoxin of " molecular dimensions " over the susceptible tissues of the skin. The tissue antitoxin must in this case be uniformly distributed throughout a uniformly reacting tissue like the guinea-pig's skin. In contrast, the distribution of intradermal toxin, injected from a single point in the skin is unlikely to be uniform ; and it will be profitable to consider what it is, and how it will affect indirect neutralization. The distribution of toxin will depend on the one hand on the absorbing power of the tissues, and, on the other hand, the outward flow of toxin solution from the needle-point first under the pressure of injection, then under the pressure of the tissues distended with liquid by the force of injection and, when these mechanical forces are spent, by diffusion and other molecular forces. On immediate injection, 0·1 ml. of fluid regularly raises a bleb 10·0 to 11·5 mm. in diameter. One hour later a flat 15 to 18 mm. bleb is recognizable under oblique illumination of the skin surface ; and after a few hours the fluid is mechanically spread over an area about 20 mm. in diameter. It is questionable how far diffusion, etc., is operative, but lesion-diameters of up to 30 mm. accord with the linear relationship described above, though they are presumably due to toxin spread by means other than the injec-

tion pressure. But in lesions up to 20 mm. in diameter, the distribution of effective toxin may be assumed to be the resultant of adsorption of toxin and mechanical dispersion of the toxin solution alone; and for this part of the curve, the molecular dispersing forces may be neglected.

It is experimentally obvious that the distribution of toxin is not uniform, because, with increasing dose of toxin, the inflammation becomes not only more intense, but disproportionately more intense in the centre of the lesion—an effect commonly seen in lesions following the intradermal injection of many toxic substances. The concentration, then, may be assumed to be highest in the centre, and in each lesion there must be finally established, from the centre outwards, a concentration gradient of toxin ending with the minimal intoxicating concentration at the periphery. There are no data for determining the shape of this gradient. It may resemble those in Fig. 6*a*, *b* or *c*, or it may be a more or less exaggerated version of one of them. It is convenient to assume a gradient with a feature common to all three, namely, a straight line, and later to consider whether conclusions derived from this assumption are valid for other hypothetical gradients.

Calculation of hypothetical linear concentration gradients.—Fig. 6*d* and *e* are median cross sections of lesions in which the concentration gradient is linear; the abscissa represents the diameter, and the ordinate the concentration of toxin. The total dose of toxin is therefore represented by a cone whose base is the area of the lesion, whose height is expressed in arbitrary units of toxin and whose volume is $\pi/3$ (area $\times$ height). Part of the toxin injected is ineffective because of the basic natural immunity of the skin tissue. The distribution of toxin in the maximum ineffective dose is represented in Fig. 6*d* by the shaded triangle whose base is $2z$, where z is the radius of the circle over which the toxin is distributed, and whose height is Ci, the maximum concentration of *ineffective* toxin at the centre of the injection site; Ci is therefore a measure of the basic natural immunity of the skin, and is uniform throughout the skin area used in the titration of toxin (p. 322). When the central concentration of toxin is $>Ci$ (Fig. 6*e*) measurable inflammation occurs; r is the radius of the lesion, and Ce is the central concentration of *effective* toxin. The maximum ineffective dose (m.i.d.) of toxin is determinable by extrapolation of the dose-response line to zero lesion-diameter, and is a convenient unit measure of toxin. From Fig. 6*d*, 1 m.i.d. $= z^2\,Ci\,.\,\pi/3$. Expressing the dose of toxin as x m.i.d., when $x>1$ we have from Fig. 6*e*

$$x = z^2\,(Ce + Ci)\,.\,\pi/3.$$

Since $\pi/3$ is a constant and nearly unity (1·047) the equation may be written

$$x = z^2\,(Ce + Ci) \quad . \quad . \quad . \quad . \quad . \quad (1)$$

The effective part (Te) of the dose x is clearly

$$Te = r^2Ce.$$

But from the figure

$$z = r(Ce + Ci)/Ce.$$

Substituting in (1) we have

$$x/r^2 = (Ce + Ci)^3/Ce^2 \quad . \quad . \quad . \quad . \quad (2)$$

In this equation x and r are determinable, z is eliminated, and Ci is by definition a constant.

As a numerical example we may take a dose-response line determined early in this work, in which a two-fold decrease in the dose x decreased the radius by 1·5 mm. By extrapolation the m.i.d. was 1/512,000 Lf. In the expression for the dose-response line, $d = b \log x = 2r$; b is therefore — 10 and $r = -5 \log x$. Since the slope b is negative only because the graphs record increasing dilution of toxin from left to right, we may omit the minus sign and write $r = 5 \log x$. Applying this to (2) we find by differentiation that the minimum value of x/r^2 is 0·391, and $Ci = 0{\cdot}058$ m.i.d. One m.i.d. of toxin is therefore $0{\cdot}058z^2$, and z, the radius of spread of ineffective toxin, is 4·15 mm. That is, 1/512,000 Lf of toxin spreads over an area 8·3 mm. in diameter.

With regard to effective doses of toxin, there is no simple solution of equation (2) to get Ce in terms of x/r^2 and Ci. The values in Table II were obtained empirically. When, for example, 1000 m.i.d. are injected, the lesion diameter is 30 mm. and 96 per cent of the dose is toxic ; with a dose of 4 m.i.d. only 14·7 per cent is toxic, and produces a lesion 6 mm. in diameter. The toxin gradients for this series of doses is illustrated in Fig. 6*f*. The most striking feature is the highly disproportionate increase in Ce as the dose increases, if the toxin is to be accommodated in lesions of the diameters observed experimentally. Ce in lesions 15 and 30 mm. in diameter is 5·5 and 65·6 times that in a lesion 6 mm. in diameter.

TABLE II.—*The Relation Between Lesion-Diameter and the Distribution of Toxin After Intradermal Injection (see Fig. 6f).*

Radius of lesion (mm.) r.	Total dose of toxin (m.i.d.). x.	Central concentration of effective toxin (m.i.d.). $x/r^2 = Ce$.	Amount of effective toxin (m.i.d.). $r^2Ce = Te$.	Percentage of x that is effective. $100(x - Te)/x$.
1	1·58	0·016	0·016	1·1
3	3·98	0·065	0·585	14·7
5	10·00	0·150	3·750	37·5
7·5	31·62	0·358	20·137	63·7
10	100	0·814	81·400	81·4
12·5	316·2	1·820	284·375	89·9
15	1000·0	4·268	960·300	96·0

For explanation of symbols, see p. 329.

The importance of this numerical demonstration, which serves mainly to emphasize the experimental commonplace of disproportionately increasing severity in the centre of skin lesion with increasing dose of toxin, lies in testing the validity of Friedemann's hypothesis of an immediately established, definitive concentration of antitoxin in the tissues. Translated into the terms used above, the hypothesis postulates that immediately after intravenous injection of antitoxin, Ci is raised uniformly throughout the skin and that no subsequent increase in Ci is effective. Thus, in the experiment summarized in Fig. 1, an intravenous concentration of 60u. antitoxin per kg. decreased the potency of the toxin 51·4-fold ; on Friedemann's hypothesis Ci was increased 51·4-fold. But it is clear also from Fig. 1 that the slope for toxin alone is almost parallel to that for toxin

indirectly neutralized by 60 u. per kg. ; the two slopes were respectively — 5·23 and — 7·45, and did not differ significantly from one another. These two statements, however, are not compatible with the relation of b and Ci in equation (2). Since $r = b/2$ (log x), we may rewrite the equation

$$4x/b^2 \log^2 x = (Ce + Ci)^3/Ce^2.$$

Both these functions have the same minimum ; and since the minimum of $4x/b^2 \log^2 x$ is a multiple of $1/b^2$, and the minimum of $(Ce + Ci)^3/Ce^2$ is a multiple of Ci, $Ci \times b^2$ is a constant, and

$$b^2 \propto 1/Ci.$$

That is, as Ci increases, b, the slope of the dose-response line, decreases. Given — 5·23 as the slope for toxin alone, the slope for indirectly neutralized toxin should, on Friedemann's hypothesis, be $-5{\cdot}23/\sqrt{51{\cdot}4} = -0{\cdot}73$, a sevenfold diminution. Now, the estimates of the two slopes in Fig. 1 are subject to error, but the regression analysis would certainly have detected as heterogeneous any slope differing by a factor even of two, from that of the dose-response to toxin alone.

Non-linear concentration gradients. The values for $Ci + Ce$ derived from equation (2), for the concentration of toxin at the centre of the lesion provide a means of testing whether toxin is absorbed to the tissues according to Freundlich's absorption isotherm. The constant absorbing surface is represented by a narrow cylinder of tissue at the centre of the lesion, and the amount absorbed is $(Ci + Ce)$. The concentration of toxin added to this system is x in the notation used above. The isotherm is

$$(Ce + Ce) = ax^n$$
$$\text{or } \log(Ci + Ce) = \log a + n \log x$$

where a is a constant and $n < 1$; and the data may be considered to fit if the relation between $\log(Ci + Ce)$ and $\log x$ is linear. Table III lists the relevant data from Table II. The plot of values in Columns 3 and 5 lie close to a straight line. The approximate value of n, however, is > 1, being 1·15 ; implying that the greater the concentration of toxin, the greater the proportion of toxin absorbed. Consequently, if the isotherm applies, the acutely peaked distributions in Fig. 6*f* are highly unlikely. To meet this difficulty we may assume that at the centre of the lesion a substantial core of tissue contains the maximum concentration of toxin. That is, the distribution is in the form of a truncated triangle, and approximates more to Fig. 6*a* or *b*.

TABLE III.—*The Absorption of Toxin at the Centre of Skin-lesions in Terms of Dose Injected. Freundlich's Absorption Isotherm.*

r (mm.).	$Ce + Ci$ (m.i.d.).	Log $(Ce + Ci)$.	x (m.i.d.).	Log x.
6	0·123	$\bar{2}$·8129	3·98	0·6
10	0·208	$\bar{1}$·1761	10·0	1·0
15	0·416	$\bar{1}$·5539	31·6	1·5
20	0·872	$\bar{1}$·9106	100·0	2·0
25	1·878	0·2601	316·0	2·5

Nevertheless, with regard to all the hypothetical concentration gradients other than the linear, it will be clear from the values of Ce in the table that a greatly disproportionate elongation of the distribution curve of toxin in the

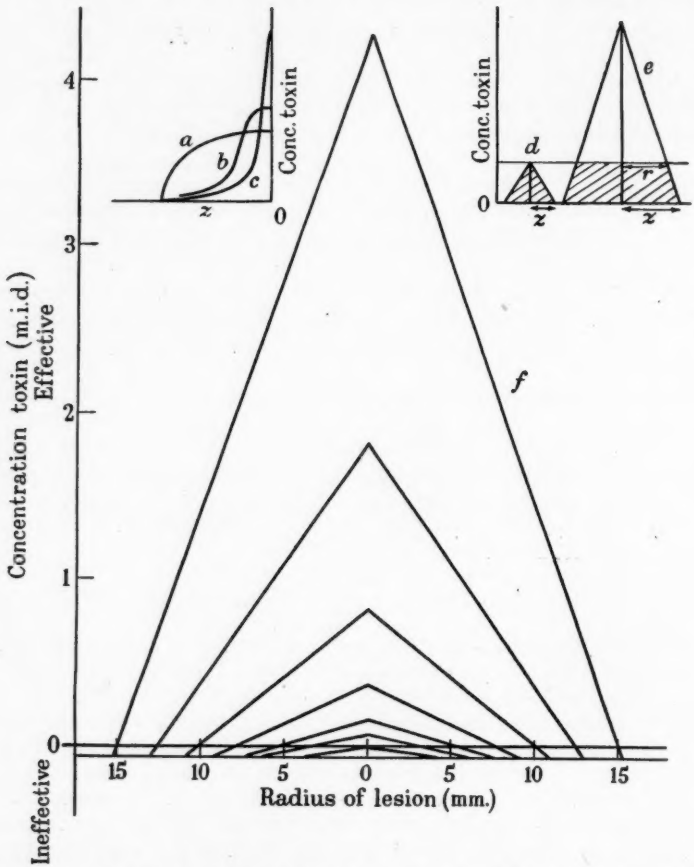

FIG. 6.—Diagrams of the distribution of diphtheria toxin in median cross-sections of skin-lesions after intradermal injection.
(*a*), (*b*) and (*c*). Possible types of the gradient of toxin concentration from the centre of the lesion.
(*d*) and (*e*). Distribution of a maximum ineffective dose (m.i.d.), and of an effective dose of toxin, with a linear concentration gradient.
(*f*) Hypothetical distribution of graded doses of toxin, with linear concentration gradients, in guinea-pig skin lesions, for a dose-response where $r = 5 \log x$ (Table II).

lesion will occur as the dose increases, whatever the shape of the distribution, and on Friedemann's hypothesis, therefore, that a decrease in slope is likely with increased Ci. It might be possible to devise a concentration gradient, different from the simplified linear gradient from which equation (2) was derived,

such that the slope of the dose-response would remain constant with increasing *Ci*. But two hypotheses are now required to explain the observed constancy of slope. Firstly, that *Ci* is uniformly raised to a definitive level soon after the injection of antitoxin—a consequence of the properties of antibody globulin and capillary endothelium; and, secondly, that the distribution of a quite different substance—diphtheria toxin—in skin tissue is precisely adjusted to compensate for any rise in *Ci* from that of normal tissues to at least (Fig. 5) 3000 times this value. It is more economical of hypotheses to assume that the degree of indirect neutralization is determined primarily by the distribution of toxin, without any assumption about the establishment of a definitive concentration of antitoxin in normal tissues.

Factors affecting indirect neutralization.

The simplest assumption is that the degree of indirect neutralization, i.e. the rate at which antitoxin passes through the capillary walls, is a function of the concentration of toxin in the tissue; in other words, that toxin stimulates the passage of antitoxin. Toxins like those of *Cl. welchii*, *Cl. septicum*, or the cobra rapidly increase the capillary permeability in the skin of the guinea-pig. In animals with pontamine sky blue 6 X (75 mg. per kg. body weight) in the circulation, these toxins in 5 to 30 minutes induced intense staining of the lesion by exuded dye-stained plasma, the speed and area of exudation being greater with higher concentrations of toxin. No such exudation occurred in diphtheria toxin lesions up to 7 hours old. In 8- to 9-hour lesions there was slight exudation of coloured plasma in irregular areas round the periphery, and 20-hour lesions were readily permeable to circulating dye. Gross capillary trauma in the first six hours may therefore be excluded.

The trauma of injection. The injection of 0·1 ml. of 0·85 per cent saline, or of Ringer's or Locke's solution, did some damage, producing a faint blue stain 3 to 4 mm. in diameter when the fluid was injected slowly in 2 to 3 seconds, and up to 5 mm. when the fluid was injected as rapidly as possible to produce maximum trauma. Since antitoxin might exude into the tissues by reason of the trauma, and affect the whole lesion, the magnitude of this traumatic leak was estimated.

In the first place it takes place mainly in the first hour after injection, because when pontamine blue was injected into animals with saline lesions one hour old, the blueing was about 20 per cent of that in lesions made in animals with already circulating dye, showing that after 1 hour the tissues had largely recovered from the injection-trauma.

Secondly, the volume of plasma leaking through is small. The mean area and intensity of blueing after 1 hour was estimated in several lesions made by injecting 0·1 ml. saline into two guinea-pigs with circulating pontamine blue. Three ml. samples of blood from both animals were then defibrinated, mixed and centrifuged, and the dyed serum diluted serially. The undiluted serum was injected intradermally into fresh animals in 0·01, 0·015, 0·02 and 0·03 ml. volumes from a microsyringe and the mean diameter of the blued areas measured to provide a standard volume-area relation. At the same time 0·1 ml. of the serum dilutions were injected to provide standards for intensity. By direct matching of these lesions with the blue saline lesions, the volume of circulating

plasma required to produce the observed degree and area of blueing by exudation or leak could be estimated ; in the two animals it proved to be 0·0013 and 0·0025 ml. respectively. These figures are reliable only as indications of the *order* of size of the plasma leakage induced by saline.

Thirdly, antitoxin in volumes similar to that of the saline leak does not affect toxin lesions. Guinea-pigs were injected with enough toxin to produce lesions 18 mm. in diameter, and immediately afterwards, 0·005 ml. volumes of antitoxin solution (representing twice the volume of the estimated saline leak) were injected with a microsyringe into the centre of the blebs. Antitoxin in concentrations up to 2000 u. per ml., which is far in excess of the plasma concentration

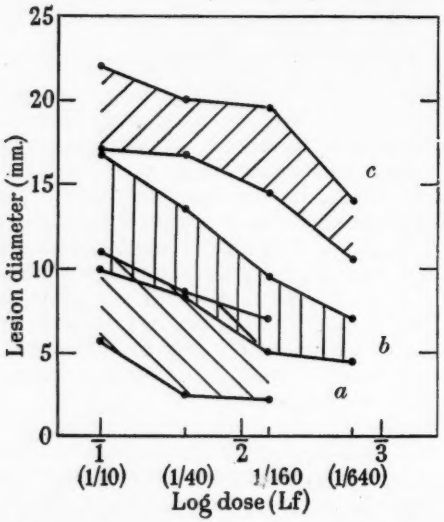

FIG. 7.—The " transient-zone " phenomenon following the intradermal injection of diphtheria toxin into guinea-pigs with circulating pontamine blue. The heights of the shaded areas represent twice the width of the zone of transient grey-blue coloration in lesions 2 hours (lower band), 3 hours (middle band) and 4½ hours old (upper band).

in indirect neutralization, failed to diminish the size of the lesion significantly. This result incidentally suggests that diffusion of antitoxin from the centre of an injection bleb is exceedingly slow, for the amount injected was in some cases sufficient to neutralize several thousand times the amount of toxin present.

For these reasons, the capillary permeability induced by the unavoidable trauma of injection is a negligible factor in indirect neutralization by antitoxin.

The " transient-zone " phenomenon. Certain changes, however, occur in the early stages of toxin lesions in animals with circulating dye. As the lesion ages, a faint but definite grey-blue colour, more intense and darker than that of the surrounding skin, develops at the centre. The area of this coloration expands, and at the same time the colour in the centre changes to a lighter blueish-pink, leaving a zone of grey-blue which expands till it reaches the edge of the lesion and then disappears. Fig. 7 epitomizes the essentials of this phenomenon of the

expanding, transient zone. The measures were made in guinea-pigs given 4 different intradermal doses of toxin at four different times, so that one hour after the intravenous injection of dye, when the readings were taken, the four titration series were respectively 1, 2, 3 and 4½ hours old. The grey-blue zone appears first after 2½ hours, in the centre of the lesion (where toxin is most concentrated); it is wider and appears earlier with the large doses of toxin, and reaches its maximum size in about 5 hours, and then gradually disappears. The phenomenon cannot be elicited easily in all guinea-pigs. Some show it in all lesions of the right age and others erratically, only in a few lesions; but in all the animals tested there was some sign of it, and it may therefore be taken as a characteristic feature of the reaction of guinea-pig skin to diphtheria toxin. It is not due to an impurity in the toxin, because it did not occur with toxin mixed with a just-neutralizing dose of antitoxin. Nor is it likely to be a subliminal effect of any substance in the toxin which produces gross capillary permeability, because the toxin was used at dilutions of 1/640, and induced gross permeability only when 1/5 (containing 15·6 Lf per ml.) was injected. The grey-blue colour was diminished by pressure, and appeared to be due to an intense transient vascular dilatation lasting about 1 hour in any one part of the lesion. The phenomenon is not the same as the "traumatic reaction" described by Jensen (1933) in rabbits, which is maximum in 6 hours with a diameter of only 4 mm.; this also was observed in the guinea-pig lesions. It must be considered in the light of vascular physiology. Between the permeability of capillaries in normal skeletal tissue, permitting a slow passage of serum protein, and the gross permeability induced, for example, by histamine, peptone, certain bacterial toxins, and by acute inflammation (Field, Drinker and White, 1932), there may be a range of permeabilities characteristic of different physiological and pathological states. Whether in the transient zone there is any increase in the transendothelial passage of antibody can be decided only by direct demonstration of increased globulin exudation in these circumstances. Nevertheless, the toxin lesion obviously exhibits a substantial vascular abnormality in its early stages, which may be a factor in indirect neutralization.

The rate of fixation of toxin in skin tissue.

Though it is tempting to regard the gradually increasing inefficacy of late-administered intravenous antitoxin as a direct measure of the rate of fixation of toxin (Glenny and Hopkins, 1925), our ignorance of changes in capillary permeability in a progressive inflammatory lesion does not warrant it. For this reason, Wright and Clark's (1944) results are important and instructive. They injected antitoxin into toxin lesions already established in the human skin, and found that lesions 8 to 12 hours old were modifiable in some degree by the antibody. It is possible to elaborate his findings in the more accessible guinea-pig. The 0·1 ml. volumes of toxin were injected through a fine needle coated on the outside with a dye and inserted in a determined direction. Through the needle-hole thus stained with dye it was possible to "superinject" antitoxin, using a slightly wider needle to avoid back leak along the old needle track, and thus to ensure a distribution of both toxin and antitoxin solutions from the same point in the skin. The technique has certain drawbacks. Firstly, because the toxin may have spread beyond the confines of the injection-bleb by the time antitoxin is superinjected, a larger volume of antitoxin solution (0·2 ml.) is

required if the antibody is to reach the limits of intoxicated tissue; and this volume of fluid, apart from its antitoxic effect, might drive unfixed toxin further from the point of injection, and create a potentially bigger lesion. In these circumstances a superinjected lesion at 24 hours, of the same size as the control lesion, might be evidence of a considerable neutralization, though apparently indicating none. To overcome this difficulty controls superinjected with saline were included in all tests.

Secondly, a lesion superinjected immediately after toxin is necessary as a reference point. In practice, the immediacy could not be approached nearer than 2 seconds, and 5 seconds in a series of injection, if a constant time interval were to be maintained. Controls were also included of injections of 0·3 ml. of the appropriate toxin-antitoxin mixture made *in vitro*, and of 0·1 ml. toxin superinjected into blebs made by 0·2 ml. antitoxin; but the conditions differed so much from the test lesions that only gross differences in results were significant.

Fig. 8, I, records the results of one test with a dose of toxin yielding a mean lesion-diameter of 19·3 mm. Superinjected with saline, the lesion-diameter was 25·8 mm.; the saline effect slowly declined as the superinjection was delayed, and was small after a 3 hours delay. This phenomenon is itself evidence against an immediate fixation of toxin, but only on the assumption that the superinjected saline was dispersing the original diphtheria toxin, and not a secondary inflaming agent produced by the action of toxin on the tissues. However, the neutralization by antitoxin superinjected at the corresponding times (lower curve) proves that the large "saline" lesion-diameter (S) is due to dispersion of the toxin itself; the "antitoxin" lesion diameter (A) is 13·6 mm. less than S, and 7·1 mm. less than the "toxin control" diameter.

Though both upper and lower curves indicate a delayed fixation of toxin, they do not reflect the rate of fixation. Neutralization by superinjected antitoxin is direct, and at first sight the difference, S — A, should lie on a dose-response curve similar to that in lower part of *b* and *c* in Fig. 2. That is, on a slope of about — 35, when the ratio (R) of potencies of neutralized and un-neutralized toxin would be antilog [(S — A)/35]. The S lesion, however, is not a valid control for this purpose. If the immediately-superinjected 0·2 ml. saline merely diluted the 0·1 ml. toxin solution already present, the resulting lesion should resemble that produced by the same amount of toxin injected in a volume of 0·3 ml. But a change from 0·1 to 0·3 ml. in the volume in which a given amount of toxin is injected does not alter the size of the lesion. For example, eight guinea-pigs were injected with four concentrations of toxin (1/400, 1/800, 1/1600 and 1/3200 Lf/ml.) each in volumes of 0·05, 0·1, 0·2 and 0·4 ml. The injection sites were partially randomized among the animals. For each combination of toxin concentration and injection-volume, the mean lesion-diameters in mm. were:

	Volume injected (ml.).			
Lf/ml.	0·4	0·2	0·1	0·05
1/400	26·9	20·4	16·5	12·5
1/800	21·3	16·1	11·5	10·5
1/1600	16·8	13·0	9·4	7·0
1/3200	11·5	10·2	7·9	4·6

Reading diagonally upwards from left to right, it will be seen that the same *amount* of toxin produces approximately the same lesion-diameter. Thus 0·4 ml. of 1/1600, 0·2 ml. of 1/800 and 0·1 ml. of 1/400 give lesions 16·8, 16·1 and 16·5 mm. in diameter. The increase in size by superinjected saline is therefore due to a mechanical displacement of toxin, which must to some extent be pushed outwards by the saline, piston-wise, along the narrow intercellular channels forced open by the injection. The concentration gradient of toxin that characterizes a single injection of toxin, and consequently the dose-response, is therefore disturbed by superinjection, so that the mean slope of — 35 is not valid.

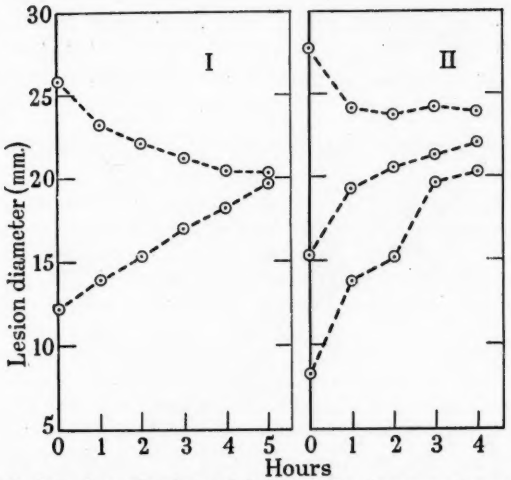

FIG. 8.—The effect in the guinea-pig of superinjecting saline (uppermost curve) and antitoxin into diphtheria toxin skin-lesions of various ages.
I. Toxin Lf 1/1000, antitoxin 0·002 u.
II. „ Lf 1/ 250, „ 0·002 u. (middle curve) and 0·128 u. (lower curve).
Each curve is the mean from three guinea-pigs, each with two sets of lesions.

But saline has little effect in lesions 2–3 hours old, so that in these the initial concentration gradient must still obtain. The value of (S′ — A) is therefore a better measure of decrease in toxicity, where S′ is the mean value of S in lesions superinjected with saline after 3 or more hours. Antilog [(S′ — A)/35] is then a better measure of R, the ratio of potencies of neutralized and un-neutralized toxin. If at each reading (0, 1, 2 . . . etc. hours) the potency of toxin in the S′ lesion is taken as unity, that in the A lesion is 1/R, and 1 — 1/R is the proportion of toxin neutralized. Putting the value of 1 — 1/R at 0 hours = a, when neutralization by superinjected antitoxin is maximum, the percentage neutralization after longer periods in terms of this maximum is $100(1 - 1/R)/a$. These values from the experiment illustrated in Fig. 8, I, are listed in Table IV.

In another experiment (Fig. 8, II) it was clear that, compared with weak antitoxin (0·02 u./ml.), antitoxin 64 times as strong (1·28 u./ml.) could affect toxin even in lesions 5 and 6 hours old. But the general effect of the two con-

TABLE IV.—*The Calculation of Percentage Neutralization by Antitoxin Superinjected into Intradermal Toxin Lesions of Various Ages (see Fig. 10).*

Superinjection after (hours).	(S′ − A) (mm.).	Ratio toxicity A and S′ lesions. R = antilog (S′ − A)/35.	Proportion toxin neutralized $p = 1 - 1/R$.	Neutralization as percentage of maximum possible $100p/0{\cdot}429$.
0	8·47	1·75	0·429	100·0
1	6·89	1·57	0·363	85·0
2	5·64	1·45	0·320	74·5
3	3·72	1·28	0·185	43·2
4	2·49	1·18	0·152	35·5
5	1·14	1·08	0·068	15·8

For explanation of symbols, see p. 337.

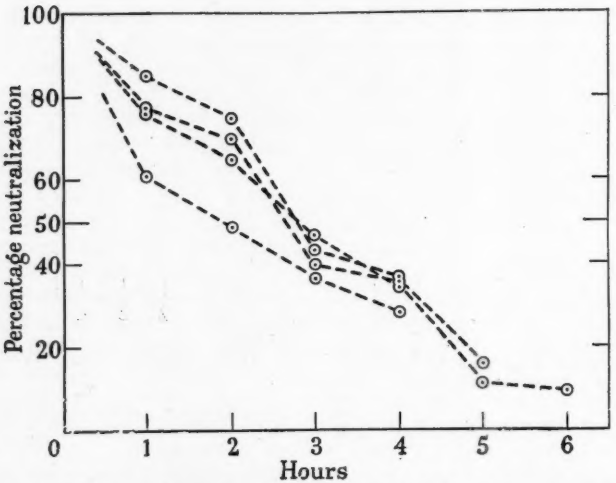

FIG. 9.—The rate of fixation of intradermally-injected diphtheria toxin, as indicated by the percentage decrease in the neutralizing potency of superinjected antitoxin with increasing age of lesion.

centrations of antitoxin is the same, as Fig. 9 shows, where the percentage neutralization from the two experiments summarized in Fig. 8, I and II, and from a third experiment with 0·02 u./ml. of antitoxin, are plotted. In all, there is in the first few hours a similar proportional diminution of neutralizing power of antitoxin with the age of the lesion at the time of superinjection, and, by implication, a roughly constant rate of fixation of toxin to the tissues. The conclusions of previous investigators (e.g. Schick, Kassowitz and Buracci, 1916; Glenny and Hopkins, 1925; d'Antona, 1937; Petherick and Singer, 1944; Wright and Clark, 1944) about the slow rate of fixation in the skin are thus confirmed. The rate in other, more susceptible, tissues is largely unknown, though the

experiments of Doenitz (1899) on the rapidly developing inefficacy of intravenous antitoxin after intravenous toxin, suggest that the skin may be exceptionally slow in this respect.

The competition for toxin between tissue and antitoxin.

The curves in Fig. 8 demonstrate that whereas the concentration gradient resulting from a primary intradermal injection can be disturbed by saline superinjected up to 3 hours later, it is susceptible to modification by antitoxin for 2 to 3 hours longer. That is, toxin that cannot be shifted mechanically can be neutralized *in situ* by antitoxin, which presumably combines with already " fixed " toxin. Three stages in fixation in the skin are distinguishable :

(1) Simple absorption of toxin to the tissues ; it takes place immediately on injection, and determines the concentration gradient of toxin in the skin, and therefore the nature of the dose-response revealed by inflammatory lesions 24 hours later.

(2) Fixation of absorbed toxin so that it cannot be reversed by a limited fowl of saline ; it is complete within 2 to 3 hours of injection.

(3) Fixation of absorbed toxin which is so firm that it cannot be reversed by antitoxin ; it is complete within 5 to 6 hours of injection. This degree of fixation is most important in antitoxin therapy.

Between Stages (2) and (3) there is clearly competition for the toxin between tissues and antitoxin. Do these three stages occur in the indirectly neutralized skin lesion, and if so, is there competition in Stage (1) ?

When intradermal toxin is completely neutralized, it is theoretically possible that the injected toxin meets sufficient tissue antitoxin to neutralize it, and that antitoxin and tissues do not compete for toxin. But it is equally possible that some, and perhaps a large part, of the toxin is neutralized by antitoxin passing through the capillary endothelium after the injection of toxin ; and in this case there would necessarily be competition between tissues and antitoxin.

In an animal with already circulating antitoxin that receives intradermal toxin, the removal of circulating antitoxin a few moments later should not affect the neutralization of the toxin if Friedemann's hypothesis is true. Any increase of lesion-diameter, compared with a control animal left with its circulating antitoxin, would suggest that neutralization continued after the injection of toxin. It may be objected that increase in lesion-diameter would in such circumstances not necessarily disprove Friedemann's hypothesis, since the antitoxin equilibrium is disturbed, so that the newly-formed toxin-antitoxin complex in the tissues dissociates, releasing toxin. But in this case it would clearly be necessary to postulate competition between tissues and antitoxin, and Friedemann postulates that there is none.

The *rapid* removal of blood from a passively immunized guinea-pig and its substitution by antitoxin-free blood is technically difficult. The recent advances in methods of purifying diphtheria toxoid provide a means of avoiding this difficulty, namely, neutralization of the circulating antitoxin with concentrated, innocuous toxoid.

Neutralization of circulating antitoxin by toxoid. A specimen of purified toxoid prepared by Dr. L. Holt was available, containing 1700 Lf per mg. N and 250 Lf per mg.; its M.R.D. was 10 Lf. It readily formed a clear solution

in 0·85 per cent saline. Its equivalence with the antitoxin used throughout the experiments was checked by constant-antigen (Ramon) titrations, and solutions were made containing 40, 80, 400 and 1200 Lf per ml. The antitoxin was used at 40 u. per ml. Circulating toxoid *per se* did not affect intradermal toxin. Toxin was titrated in 9 guinea-pigs ; five minutes after the injection of the toxin three received 1·5 Lf toxoid per kg., intravenously, and three received 15 Lf per kg. The dose-response curves from all three groups were linear, parallel and substantially coincident.

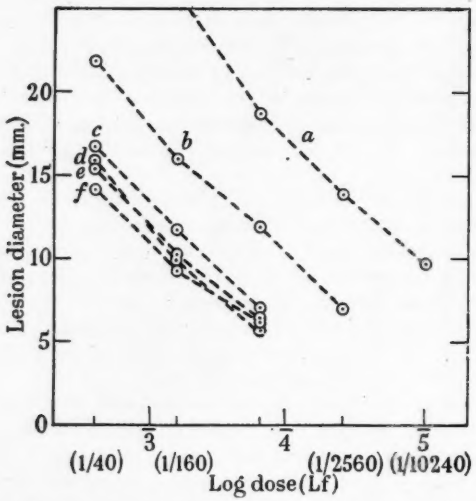

FIG. 10.—The effect of intravenous toxoid on the indirect neutralization of intradermal diphtheria toxin by circulating antitoxin.
(*a*) Antitoxin *nil* ; toxoid *nil*.
(*b*) Antitoxin 60 u. per kg. ; ten " equivalents " of toxoid after 2 minutes.
(*c*) As (*b*), but the toxoid after 1 hour.
(*d*) and (*e*). As (*b*), but the toxoid after 2 and 3 hours.
(*f*) Antitoxin alone.

The " deviation " of circulating antitoxin by toxoid was tried in guinea-pigs given 60 u. antitoxin per kg., and intradermal toxin 3 to 5 minutes later. Two minutes after the toxin either 1, 2, 10 or 30 equivalents of toxoid were injected intravenously. In other tests, the toxoid and antitoxin were mixed *in vitro* before injection. The results were as follows.

One or two equivalents of toxoid given 2 minutes after toxin slightly diminished the efficacy of the indirect neutralization ; ten equivalents diminished it significantly (Fig. 10*a*, *b* and *f*), but 30 equivalents were no better. The curves *c*, *d* and *e* in Fig. 10 show the decrease in the toxoid effect with increasing delay in injecting it after the toxin.

At first sight these results are compatible with Friedemann's hypothesis, because one equivalent, presumably neutralizing all circulating antitoxin, did not affect the skin toxin appreciably, and the ten equivalents may have done so by passing from the circulation into the tissues and disturbing the toxin-

antitoxin complexes already formed there (*cf.* the modifying effect of toxoid added to toxin-antitoxin mixtures *in vitro*; Schmidt and Scholz, 1929). There is, however, good evidence that the one equivalent of toxoid did not neutralize all the circulating antitoxin. Thus, 10 equivalents of toxoid diminished the efficacy of antitoxin to the same extent when given just *before* the toxin, and when mixed with antitoxin *in vitro*, and injected either immediately, or after standing for 1 hour at 37° C. That is to say, the combination between 10 equivalents of toxoid and antitoxin, whether taking place *in vivo* or *in vitro*, was incomplete in the sense that some antitoxin was available *in vivo* for the neutralization of tissue toxin. Moreover, since the degree of neutralization was the same in all cases, the effect of toxoid before or after toxin was the same as the injection in the first place of a smaller amount of antitoxin. We must therefore conclude that, owing perhaps to slowness of combination and differences in the rates of removal of toxoid and antitoxin from the circulation, one equivalent of toxoid did not neutralize *in vivo*; and that 10 equivalents acted by neutralizing the circulating antitoxin, and not by penetrating the tissue and modifying any toxin-antitoxin complexes formed therein.

Reading off the 14 mm. lesion-diameters in Fig. 10, the toxic doses in Lf for no antitoxin (*a*), antitoxin + toxoid (*b*) and antitoxin alone (*f*) are 1/2500, 1/320 and 1/40. In this instance the toxoid has diminished the antitoxic potency by half; so that at least half the neutralization in the skin lesion took place *after* the injection of toxin.

DISCUSSION.

The neutralization of intradermal toxin is only remotely analogous to antitoxin therapy of natural diphtheria. Nevertheless, the skin lesion is peculiarly apt for investigation of the problems of antitoxin therapy. It is surprisingly regular, and the regression line of log dose upon lesion-diameter is linear and provides a measure of antitoxic efficacy whose statistical significance can readily be assessed. The method is a logical extension of the "multiple Schick test" of Glenny, Hopkins and Pope (1924); in addition to the end-point determined by such a titration the dose-response curves yield numerical information about the fate of the incompletely neutralized dose of toxin, as well as about the neutralized dose. The end-point is in fact a limiting case; but without information about the conditions of which it is a limit, it cannot be considered representative of the toxin-lesion in general. I do not suggest that all diphtheria immunity should be measured by the response to graded doses of toxin, but the method deserves consideration when matters of special immunological interest are in question.

Friedemann based his conception of the rapidly-established definitive concentration of tissue antitoxin upon the following arguments:

(1) Intradermal toxin is neutralized by circulating antitoxin in constant proportions. It cannot therefore have combined with the tissues.

(2) The proportionality is disturbed when the antitoxin is given as little as two minutes after the toxin. Toxin and antitoxin must, therefore, combine within at most two minutes.

(3) The degree of indirect neutralization is the same when the antitoxin is given two minutes and 3 hours before the toxin. The tissue concentration

of antitoxin must therefore be constant over a period of hours, and rapidly established, at least in a molecular zone surrounding the susceptible cells.

(4) It follows that the ratio of indirect and direct neutralizing doses of antitoxin (*Ai:Ad*) is a valid measure of the relative concentration of antitoxin in the blood capillaries and the tissues.

I have confirmed Friedemann's findings in (1) and (3) but not in (2). The disturbance of proportionality, and any difference in the nature or degree of neutralization by antitoxin given a few minutes before and after toxin, are undetectable when small doses of toxin are used. It does not therefore follow that all effective combinations of toxin and antitoxin must take place within a few minutes.

With regard to (1), Friedemann's conclusion is not inevitable. Thus, if tissues and antitoxin competed for toxin; if fixation were proportional to the concentration of toxin; and if reversal of tissue-intoxication were proportional to the concentration of antitoxin continually passing from the circulation to the tissues, proportionality might hold equally well.

The deduction from (3) may be correct; but the equilibrium so established is not necessarily definitive in the sense that the resulting concentration of tissue antitoxin at the moment of injecting toxin finally determines the degree of neutralization. It is highly unlikely that in the normal animal the equilibrium is static; because, whether antitoxin is fixed to the tissues or not, when the tissues are saturated, excess antitoxin will be removed *via* the lymphatics and its place taken by antitoxin from the blood (Drinker and Yoffey, 1941). But if the equilibrium is dynamic, then the tissue concentration at the moment of injecting the toxin can be definitive only if the toxin is rapidly fixed to the tissues, and reversal of the fixation by antitoxin is impossible. As the dose-response to toxin shows, toxin is rapidly *absorbed* to the tissues, otherwise the lesion-diameters made by any dose of toxin above a certain concentration would tend to equal the limits of spread of the injection fluid; it is not, however, rapidly *fixed*, for fixation that is irreversible by saline in a 2-hour-old lesion may to some extent be reversed by antitoxin in lesions up to 6 hours old.

Friedemann's contention that the tissue antitoxin is confined to a protective molecular zone round the cells, though it serves to explain the rapid establishment of equilibrium, does not affect objections to the hypothesis based on the demonstration of competition. It is far more likely that intradermal toxin is neutralized progressively by antitoxin continuously passing across the capillary endothelium. The transendothelial flow may be constant in the normal animal. There is, moreover, no evidence that during the first five hours of a lesion when the fixation of toxin takes place, diphtheria toxin induces a pathological increase in capillary permeability as the α-toxins of *Cl. welchii* and *Staph. aureus* do; though after two hours the flow of antitoxin may be accelerated by a physiological response of the skin vessels.

The moment the flow of antitoxin ceases to be effective will depend on the rate of fixation of toxin to the tissues, the concentration of antitoxin entering the tissues and the relative avidities of tissues and antitoxin for toxin. Diphtheria toxin is a slowly-acting capillary poison; though when it is injected into passively immunized animals together with substances that increase capillary permeability, the efficacy of the circulating antitoxin is greatly increased (Friedemann, Traub and Langstadt, 1946). The strikingly low efficacy of indirect

neutralization of tetanus toxin (Friedemann, Zuger and Hollander, 1939*c*) might well result from an even lesser toxicity for capillary endothelium, and the notoriously rapid fixation of toxin to neural tissue.

The inaccessibility of diphtheria toxin in the tissues to circulating antitoxin is at first mechanical, then pharmacological. The shortest period after which late intravenous injection of antitoxin will fail to neutralize intradermal toxin is important in antitoxin therapy but it is not a valid measure of the rate of fixation of toxin. The fact that intravenous antitoxin is largely ineffective given after 2 hours does not mean that the 2-hour-old lesion cannot be affected, but that the summation of antitoxin passing into the intoxicated tissue necessary for neutralization is not reached in a certain critical time, $(2 + x)$ hours. The value of x is uncertain, but the results of superinjecting antitoxin suggest that x is greater than 1 hour, but not much more than 4 hours.

Though a rigorous experimental disproof of Friedemann's hypothesis is wanting, the demonstration of competition in the tissues and all that it implies, contradicts some of its postulates; and the analyses of the dose-response to toxin both alone, and directly and indirectly neutralized, are difficult to reconcile with it. It follows that in the absence of data about rates of toxin fixation and transendothelial passage of antitoxin, and about the reversibility of the tissue-toxin combination, Friedemann's *Ai:Ad* ratio cannot yield any precise information about the equilibrium values of the concentration of antitoxin inside and outside the blood vessels of the normal animal; and in any event, the *Ai:Ad* ratio is formally invalid, since its magnitude depends on the response level chosen for intersecting two statistically heterogeneous dose-response lines.

SUMMARY AND CONCLUSIONS.

When diphtheria toxin is injected intradermally into a guinea-pig it is absorbed immediately to the skin tissues. A concentration gradient is thus established, declining outwards from a central maximum value depending on the dose of toxin, such that the diameter of the resulting inflammatory lesion at 24 hours is proportional to the logarithm of the dose of toxin injected. The resulting dose-response curve provides a measure of immunity that is more informative than the end-points determined by the usual methods of titration of toxin, and is susceptible to precise statistical analysis.

Immediately after injection the toxin is lightly held by the skin-tissues and can be washed away by saline. Within 2 to 3 hours all the toxin is fixed too firmly to be removed by saline, but can still be neutralized to some extent by antitoxin. That is to say, there is a degree of combination of toxin and tissue that can be reversed by antitoxin. Antitoxin was not demonstrably effective after 5 to 6 hours.

During the first six hours, fixation that is irreversible by antitoxin proceeds at an approximately uniform rate; there is little evidence that in this time the toxin induces any pathological increase in the permeability of the blood capillaries.

It appears that in the normal guinea-pig, intravenously injected antitoxin passes at a regular rate through the tissues into the lymphatic system, and there is thus established a dynamic equilibrium across the capillary endothelium. The equilibrium concentration in the tissues at the time of an intradermal dose

of toxin neutralizes only part of that dose; at least half may be neutralized by antitoxin passing into the tissues after the injection of toxin.

The moment at which circulating antitoxin ceases to be effective is therefore dependent on the rate of its passage across capillary endothelium, and the rate of fixation of toxin to the tissues. In the indirect neutralization of intradermal toxin, the moment occurs 2 to 3 hours after the injection of toxin. Since, however, the time interval is the resultant of two opposing rates, it is not a valid measure of the rate of fixation of toxin; and for the same reason, the ratio of the amounts of antitoxin neutralizing a standard dose of toxin by the indirect route and by direct mixing *in vitro*, is not a valid basis for estimating the equilibrium values for the concentration of antitoxin inside and outside the blood capillaries of the normal animal.

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THE NATURE OF ANTISTREPTOLYSIN "S" IN THE SERA OF MAN AND OF OTHER SPECIES: ANTISTREPTOLYSIN TITRES IN NORMAL AND DISEASED STATES.

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THE study of haemolysins produced by streptococci and of antibodies against them was greatly clarified by Todd (1938), who first clearly differentiated streptolysin "S," a serum-soluble, oxygen stable haemolysin, from streptolysin "O," which is readily formed in serum-free media and is inactivated by oxygen. It has been shown by him (Todd, 1932), and by many workers since, that streptolysin "O" is a powerful antigen, and that infection with strains of streptococcus which produce this haemolysin is generally followed by a sharp rise in the antistreptolysin "O" content of the serum. Streptolysin "S," on the other hand, does not readily give rise to antibodies, and although sera from various animals have long been known to contain inhibitors of "streptococcal haemolysin" (Lyall, 1914; McLeod and McNee, 1913), no increase in inhibitory action was observed after injection of haemolytic streptococci. Todd, however, claimed that by prolonged courses of intravenous injections of living Group A streptococci it was possible to obtain antisera in rabbits to streptolysin "S," that such antisera were specific for streptococci of this group, and that antibodies against streptolysin "S" were distinct from those against streptolysin "O" (Todd, 1938, 1939; Herbert and Todd, 1944). In 1939 Todd, Coburn and Hill published a study of the antistreptolysin "S" and "O" titres in normal adults, and in children with haemolytic streptococcal infections with and without rheumatic fever. The variations in antistreptolysin "S" titre were not large, but from a statistical analysis of their data these authors concluded that in response to infection with haemolytic streptococci there is a rise in the antistreptolysin "S" titre considerably above the normal level in the sera of all groups studied, with the exception of those children who developed clinical signs of rheumatic activity. Furthermore, during rheumatic attacks the antistreptolysin "S" titres tended to be lowest when the clinical symptoms were most pronounced.

Since antibodies to other streptococcal products (e.g. streptolysin "O," streptokinase, hyaluronidase, proteinase) have been shown by other workers to be increased in the sera of patients with rheumatic fever in much the same way as in the sera of persons with streptococcal infections, but without clinical rheumatic activity, it seemed that the antistreptolysin "S" response might be a clue to some significant difference between the rheumatic and the non-rheumatic groups. A limited study of such sera, which is reported below, showed that nearly all sera taken from sick persons had lower anti-streptolysin "S" titres

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than those from normal people, and failed to reveal the difference found by Todd, Coburn and Hill (1939) between those streptococcal infections which were accompanied by clinical rheumatic fever and those which were not. The investigation was extended therefore to attempts to produce immune sera to streptolysin " S " in rabbits and a horse, both by the injection of living streptococci and by potent filtrates of yeast nucleic acid broth cultures (Okamoto, 1939). These attempts were not successful, and since an examination of sera from several species of animal revealed considerable amounts of antistreptolysin " S " in cases where any streptococcal infection could be excluded, the status of antistreptolysin " S " as an antibody in the accepted sense of the word is called in question. In this paper are presented the results of antistreptolysin " S " investigations in untreated sera.

MATERIALS AND METHODS.

Sera.

Sera were obtained fresh and were stored without preservative at — 20° C. No alteration in antistreptolysin titres were observed during several months under these conditions.

Streptolysin " S."

Two strains of Group A streptococcus were used for production of streptolysin " S." During the earlier stages the strain used (NY5) was a Type 10 streptococcus, which produced powerful streptolysin " S " and some streptolysin " O." It was grown at 37° C. overnight in a beef heart infusion broth containing 10 or 20 per cent horse serum. The cocci were centrifuged down and were extracted with horse serum, thereby giving a powerful haemotoxin, which was stored in ampoules at — 76° C.

During the later stages there became available a Type 11 strain (Blackmore) which had previously been shown by Todd to produce streptolysin " S " but not " O." This strain was grown overnight at 30° C. in 20 per cent serum broth, and the euglobulin fraction of the supernatant fluid, which contained the bulk of the streptolysin activity, was precipitated according to the method described by Herbert and Todd (1944). The precipitate was dissolved in borate buffer saline pH 8·0, and stored in ampoules at — 76° C. At this temperature both the above preparations were stable for at least 6 months, although at — 20° C. or higher they rapidly declined in potency.

Towards the end of this investigation it became desirable to have a preparation of streptolysin " S " free from serum, in order to eliminate possible effects of the serum moiety. Use was made of the observation of Okamoto (1939), recently amplified by Bernheimer and Rodbart (1948), that in the presence of yeast nucleic acid large amounts of oxygen stable streptolysin are found even in the absence of serum. Strain Blackmore grown for 24 hours in infusion broth containing 1 per cent yeast nucleic acid yielded a potent haemolytic filtrate, which was reasonably stable at — 20° C. or at 4° C. As will be shown below, this filtrate was serologically equivalent to haemolysin produced in serum broth.

Finally, for comparison, a strain of group C streptococcus (Loewenthal M) was grown in 20 per cent horse serum broth in the same manner as the Group A Type 11 strain, and from it the haemolytic euglobulin fraction was also prepared.

Titration of antistreptolysin " S."

The method used was that given by Todd (1938), in which the unknown serum is titrated against a sample of streptolysin which has been standardized against a standard serum at the time of test. The estimations were carried out in borate buffer saline, at pH 8·0, and rabbit erythrocytes were used as indicators. It was found that a suitable amount of streptolysin was that which caused 50 per cent lysis of the erythrocytes in 2 hours under standard conditions. A normal human serum was set aside as a standard ; it was kept stored at — 20° C. To it was assigned an arbitrary value of 10 units antistreptolysin " S " per ml. Although this standard was not the same as Todd's standard, it was probably fairly close, since the range of antistreptolysin titres found did not differ greatly from his previous records.

Titrations of antistreptolysin " O."

These were done by Todd's (1938) method, modified by use of an isotonic phosphate buffer pH 6·5 for dilutions and of M/100 thiolacetic acid for reduction as recommended by Herbert and Todd (1941).

Immunization of experimental animals.

Rabbits.—Six rabbits of mixed breeds were given intravenous injections of Streptococcus Blackmore grown in 20 per cent rabbit serum broth, and suspended in the supernatant fluid. The first two injections consisted of killed organisms, but all later injections contained living streptococci. Injections were given 2 or 3 times weekly, with rest periods of a fortnight or more between courses. The total volumes of packed living organisms injected varied from 0·6 to 1·0 ml., and of culture fluid from 12 to 20 ml. Serum samples were taken at the beginning, in the middle, and one week after the end of each course.

Horse.—The course of immunization was kindly performed by Dr. C. L. Oakley, at the Wellcome Research Laboratories, Beckenham. Five intramuscular doses of formolized culture filtrate from streptococcus Strain Blackmore grown in yeast nucleic acid broth were followed by a total of nearly 3 l. of untreated filtrate, in 26 doses over a period of 15 weeks. Test bleedings were made weekly.

RESULTS.

Antistreptolysin " S " in human sera.

The summarized results of estimations on pathological and normal human sera are given in Table I, from which it appears that the highest antistreptolysin " S " titres were found in normal adults and in late convalescent cases of rheumatic fever. During the acute phases of streptococcal and other infections, and during the early stages of convalescence, the antistreptolysin " S " titres were comparatively low. It is also apparent that antibodies against streptolysins " S " and " O " behaved independently.

The lowest antistreptolysin titre encountered (not included in the table) was 2·7 units/ml. in the serum of a patient with steatorrhoea who was on a fat-free diet, and the highest value was 15 units/ml. in the serum of a patient with Type II nephritis and raised serum lipoids.

TABLE I.—*Antistreptolysin "S" Titres (units/ml.) in Human Sera.*

The mean antistreptolysin "O" titres are given for comparison.

Disease.	No. of sera.	Antistreptolysin "S." Range.	Antistreptolysin "S." Mean.	Antistreptolysin "O." Mean.
Scarlet fever—acute	8	3·2–8	6·3	35
Convalescent (14–16 days)	8	4–8	6·2	240
Streptococcal pharyngitis—acute	4	4–6·7	5·2	410
Subacute and convalescent	4	2–9·5	6·1	240
Rheumatic fever—acute	8	5–6·7	6·3	428
Subacute	16	4–10	6·7	261
Convalescent	15	5·3–12·5	8·4	275
Rheumatoid arthritis—acute	4	5·3–9·5	6·5	92
Subacute	4	4–7·8	6·3	130
Normals	14	8–12·5	9·7	48

Antistreptolysin "S" in the sera of other animals.

In Table II are given antistreptolysin "S" titres of various mammals, of representative teleost and elasmobranch fishes and of a marine invertebrate. The marine animals were included because they are not known to be subject to streptococcal infections, and it was of interest to learn whether their sera would inhibit streptolysin "S." The high titres found in normal rat and guinea-pig sera are noteworthy.

TABLE II.—*Antistreptolysin "S" in the Sera of Various Animal Species.*

Species.	No. of sera.	Antistreptolysin "S" (units/ml.). Range.	Antistreptolysin "S" (units/ml.). Mean.
Normal human	14	8–12·5	9·7
Horse	7	10–27	16
*Horse (*Cl. welchii* antitoxin)	6	5–7	5·5
Rabbit	12	3·2–8·1	6·6
Rat	2 pools (36 sera)	50–60	55
Guinea-pig	4 pools (12 sera)	32–56	43
Ox	1	..	23
Sheep	1	..	12
Plaice (*Pleuronectes*)	2 pools	48–50	49
Dog fish (*Scyllium*)	1 pool	..	5
Spider crab (*Carcina Maia*)	1 pool (whole blood)		1·2

* These sera were preserved with 0·3 per cent tricresol. It was found that this preservative caused a fall of 25 per cent in the antistreptolysin "S" titre in one week at + 4° C.

Correlation between antistreptolysin " S " titres using different sources of streptolysin " S."

Duplicate estimations were made on a number of sera, both of human and of other species, covering a wide range of antistreptolysin titres, in order to determine whether significant differences in titre would be obtained when streptolysin " S " from different sources was used. The results (Table III) showed that no significant differences were found between streptolysins prepared in different ways from Group A streptococci, or between streptolysins from a strain of Group A and a strain of Group C streptococci.

The apparent serological identity between yeast nucleic acid broth streptolysin and serum broth streptolysin is interesting, since in chemical properties the partially purified streptolysins, although superficially different, both appear to have properties of lipo-nucleoproteins.

Immunization experiments.

The results of the rabbit experiments are given in Table IV. No significant increase in antistreptolysin " S " titres was observed. During periods when the rabbits were seedy owing to the injections, and were losing weight, the titres tended to be lower than during rest periods. That the animals were capable of producing antibodies was shown by measurement of the streptococcal agglutinins, which reached titres of more than 1:100,000. It is of interest to record, in confirmation of Herbert and Todd (1944), that the Strain Blackmore did not cause a rise in antistreptolysin " O," and that presumably not even traces of streptolysin " O " are elaborated by this organism.

In the horse the antistreptolysin " S " titre rose from an initial value of 9 units/ml. to 20 units/ml., but at the end of the course it was only 5 units/ml. These titres lie within the range found for normal horses, though the last is unusually low.

DISCUSSION.

An antibody in the commonly accepted sense of the term is a serum component which combines with the antigen and is produced in response to the introduction of the antigen into the tissues of the organism. It is a matter of observation that antibodies are proteins, and that they are found in the β- or γ-globulin fraction of the serum. In the investigation reported antistreptolysin S failed to conform to this definition of antibodies. It was found to occur in high titre in the sera of normal animals, in the complete absence of any evidence of either present or past streptococcal infection. It could not be produced either in rabbits or in a horse by intensive courses of " immunization " with streptolysin S. Furthermore, as will be shown in a subsequent publication, the antistreptolysin S activity of serum is not predominantly associated with the β- or γ-globulin fractions. For these reasons it is considered that antistreptolysin S is not a true antibody. It may well be associated with a normal constituent of serum which fluctuates in amount irrespective of streptococcal infection. An analogous situation is presented by the non-specific substances in many sera which inhibit the influenza virus haemagglutination reaction, and which fluctuate from time to time without known cause (Smith and Westwood, 1949). If individual fluctuations depend in some degree upon metabolic activity, illnesses of different types, including streptococcal infections, might be expected to produce similar

TABLE III.—*Correlation Between Antistreptolysin Titres of Sera when Assayed Against Streptolysin "S" from Different Sources.*

	Source of streptolysin.	No. of sera.	Nature of sera.	Antistreptolysin (units/ml.). Range.	Antistreptolysin (units/ml.). Mean.	S.D. of difference.
(1)	Euglobulin ppt. from Group A, Type 11	25	Human	4–12·5	7·34	0·31
	Serum extract Group A, Type 10	25	,,	4–11·5	7·33	
(2)	Euglobulin ppt. from Group A, Type 11	14	Human and other animals	2·5–50	12·8	1·8
	Yeast nucleic acid broth filtrate from Group A, Type 11	14	..	2–56	13·9	
(3)	Euglobulin ppt. from Group A, Type 11	11	Human and rabbit	4–25	11·2	1·4
	Euglobulin ppt. from Group C	11	..	3–25	12·6	

TABLE IV.—*Antistreptolysin " S " Titres in Sera of 6 Rabbits Given Repeated Intravenous Injections of Living Hæmolytic Streptococci.*

	Antistreptolysin " S " (units/ml.).		Mean weight (kg.).
	Range.	Mean.	
Preliminary	5–8·1	6·1	3·0
Middle of first course	2·7–3·2	3·1	2·7
End of first course	2·9–4·3	3·6	2·8
,, second course	3·1–6·0	4·9	2·7
,, third course	2·5–6·7	5·0	2·9

effects on antistreptolysin S levels, but the degree might depend upon the stage, activity and duration of the illness. This could account for the observation of Todd, Coburn and Hill (1939) on sera from cases of rheumatic fever, and our own failure to confirm any correlation between antistreptolysin S level and type of disease.

The present work failed to provide any explanation of Todd's results (1938), in which he obtained evidence of an immune response to streptolysin S in rabbits by the inoculation of living streptococci.

SUMMARY.

Antistreptolysin " S " titres have been measured in sera from human beings with streptococcal infections with and without rheumatic fever, from various mammals and from representative marine animals.

The level of antistreptolysin " S " was not specifically correlated with streptococcal infection.

No evidence was obtained of increase in antistreptolysin titre after prolonged immunization of rabbits and a horse with living haemolytic streptococci or haemolytic culture filtrates.

Streptolysin " S " produced in serum broth is serologically indistinguishable from streptolysin produced in yeast nucleic acid broth without serum.

It is suggested that antistreptolysin " S " is not normally an antibody in the accepted sense of the word.

My thanks are due to Miss M. E. Smith for much technical assistance, to Dr. C. L. Oakley for carrying out the immunization of a horse and for providing horse sera, and to Mr. D. K. Hill of the Marine Biological Association Laboratories, Plymouth, for the sera of marine animals. Throughout the work I enjoyed the hospitality and advice of Professor Wilson Smith and his department.

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THE EFFECT OF SEVERAL DILUENTS ON PROTHROMBIN ACTIVITY CURVES.

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WITH the increased use of anticoagulant drugs more attention has been focused on blood coagulation methods, especially the prothrombin test. Many variations in performing the test and computing the results have been proposed, which have tended to confuse proper evaluation of the results. Several attempts, however, have been made to standardize prothrombin test procedures, and these have shown Quick's one-stage method to be accurate and easily adaptable to routine testing (Aggeler, Howard, Lucia, Clark and Astaff, 1946; Fisher, 1947, 1948; and Martin, Curfman and Cavano, 1947).

Several modifications of Quick's method are used in which the plasma is diluted to either 50 or 12·5 per cent before performing the test. The use of diluted plasma and its sources of error have been discussed in previous communications (Fisher, 1947, 1948). However, some laboratories prepare serial dilutions of normal plasma in order to standardize their thromboplastin preparations. This is not necessary when Quick's formula for the hyperbolic curve is used (Fisher, 1947, 1948).

The use of these hyperbolic " prothrombin activity curves " for standardizing thromboplastin reagent has led to some discussion as to the nature of the plasma diluent which should be used. Physiologic sodium chloride solution (0·9 per cent) is used most widely. However, this practice has been criticized by those who feel that prothrombin-free plasma is the only proper diluent. The present study was made, therefore, to note the effect of several different diluents for plasma in the preparation of these activity curves.

PREPARATION OF REAGENTS.

(1) Triple distilled water.

(2) Physiologic saline solution (0·9 per cent NaCl, reagent grade).

(3) Normal blood serum. Blood was drawn by venepuncture and allowed to clot in the tube. It was then centrifuged at moderate speed for 30 minutes and the supernatant serum withdrawn for use.

(4) Protein-free filtrate of blood serum. The serum proteins were precipitated with tungstic acid (Levinson and MacFate, 1946) and the solution was filtered. The filtrate was neutralized to approximately pH 7 with N/10 sodium hydroxide. Control: The protein-free filtrate prepared in this manner gave a negative biuret test, as compared to a portion of the original serum, which gave a positive biuret test.

(5) Adsorbed (prothrombin-free) plasmas.

(*a*) Barium plasma. The prothrombin was adsorbed on a 30 per cent suspension of barium sulphate, according to the method suggested by Tuft and Rosenfield (1947).

(*b*) Alumina plasma. A first preparation was made by the general formula of 1 volume of commercially prepared aluminium hydroxide gel to 8 volumes of citrated plasma. It was incubated for 15 minutes at 37° C., then centrifuged, and the supernatant plasma drawn off for use. This preparation was unsatisfactory, as evidenced by a preliminary test applied to all of the adsorbed preparations before use. One drop of the adsorbed plasma to be tested was placed on a glass slide and one drop each of thromboplastin and calcium chloride were added. If the fluid clotted (usually within twenty seconds), the prothrombin had not been adsorbed.

A different alumina preparation was then prepared, according to the method of Quick (1935), using a thicker aluminium hydroxide cream. This latter plasma was acceptable for use.

(*c*) Magnesium plasma was suggested. However, recent work by Maltaner (1948) has shown that the presence of magnesium ions enhances the rapidity of clotting at lower concentrations of calcium ion. Therefore it was considered not desirable to add these ions to the preparations, especially when dilution would decrease the level of calcium ions.

Tocantins (1944) has shown that some of the prothrombin and fibrinogen can be adsorbed on asbestos fibres, leaving the concentration of antithrombin unchanged.

(6) Plasma. Blood was drawn from normal healthy dogs and oxalated in the proportion of 1 part of M/10 sodium oxalate to 9 parts of blood. The blood was centrifuged, and the supernatant plasma used as the source of prothrombin-containing plasma for the tests.

(7) Thromboplastin was prepared from acetone-dehydrated rabbit brain (Difco), and M/40 calcium chloride was used to recalcify (Fisher, 1948).

EXPERIMENTS.

Barium plasma was selected as representative of the adsorbed plasmas because of the excellent results reported by Tuft and Rosenfield (1947). Quick (1935) has found alumina plasma to be consistently good. Magnesium plasma was considered to be not desirable, as stated above.

All reagents were freshly prepared and stored in the ice box until used. They were then allowed to come to room temperature. Dilutions of the normal oxalated dog plasma were made, with each diluent, in the order of 100, 70, 50, 40, 30, 20 and 10 per cent. Two determinations by Quick's method (1940) were made at each dilution on each sample of plasma. The average of the two determinations was then taken, and the figure plotted (Fig. 1).

DISCUSSION.

The prothrombin time for undiluted plasma was much shorter than is usually encountered with human plasma, since dog plasma contains a greater concentration of prothrombin (Quick, 1935). When normal dog serum was used as a

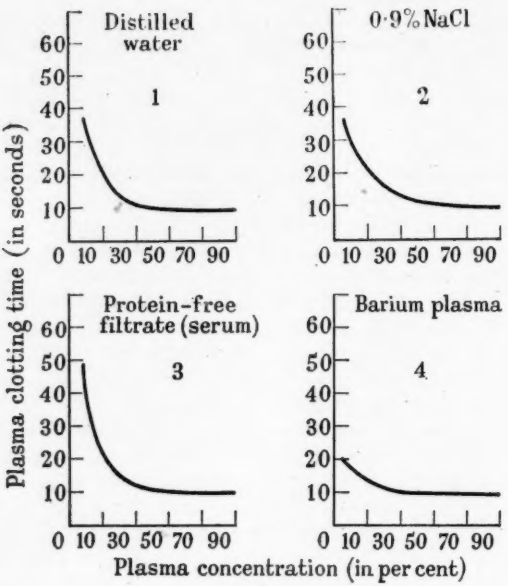

FIG. 1.

diluent, the diluted preparations were clotted within the original tubes before they could be used. This was due to the presence of small but sufficient amounts of calcium ions, prothrombin, fibrinogen and activator substances in the serum. Preparations made with barium sulphate-adsorbed plasma gave shorter prothrombin times and a somewhat more flat curve than the others. This was believed to be due to incomplete adsorption of the prothrombin, as well as the presence of other protein and ionic substances. If barium sulphate plasma is to be used, the technician must be sure that it is a pure, thick preparation and that adequate adsorption has been obtained. Owren (1947) has shown that addition of prothrombin-free (adsorbed) plasma enhances the coagulability of pathological plasmas. Saline solution, distilled water, and protein-free filtrate of serum gave results which were all similar. The characteristic hyperbolic curve was obtained in all cases. The times obtained with these preparations were all within the mean standard deviation for each point on the dilution curve, as previously calculated (Fisher, 1947).

CONCLUSIONS.

From the results obtained in these experiments it is seen that the essential change produced by serial dilution of normal plasma is reduction in the amount of prothrombin present. Distilled water, 0·9 per cent saline solution and protein-free filtrate of normal serum produced uniformly similar results as diluents, duplicating the characteristic hyperbolic curve. Prothrombin-free (adsorbed)

plasmas may be sources of error if a good preparation is not made. Owren (1947) has shown that addition of prothrombin-free plasma to normal plasma shortens the prothrombin time by Quick's method. Normal serum could not be used as a diluent due to the presence of small amounts of prothrombin, fibrinogen and activator substances.

Nitshe, Gerarde and Deutsch (1947) and Owen and Bollman (1948) recommend 0·3 per cent fibrinogen solution as a diluent, claiming that the end point is thereby made sharper at the higher dilutions. This would be favourable at the lower concentration end of the curves, or if only 12·5 per cent plasma were being used. However, the addition of extra amounts of proteins and neutral salts to whole plasma can only serve to upset the delicate colloidal balance between the reactants of the blood coagulation mechanism.

SUMMARY.

An experimental study has been presented in which was shown the effect of several diluents on "prothrombin activity curves." Normal serum was shown to be a poor diluent due to the presence of small amounts of prothrombin, fibrinogen and activator substances. Adsorbed plasma may be a source of error if a proper preparation is not made. Distilled water, 0·9 per cent sodium chloride solution and protein-free filtrate of normal serum showed uniformly consistent results, reproducing a smooth hyperbolic curve.

The author wishes to express his appreciation to Dr. Samuel A. Levinson for his interest in this work, and for allowing full use of his laboratory facilities.

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NITRATASE ADAPTATION: AN APPARENT EFFECT OF PENICILLIN WHICH CAN BE REVERSED BY SALT.

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GRAM-POSITIVE bacteria concentrate certain amino-acids in the free state in the internal environment prior to metabolism (Gale, 1947; Taylor, 1947). The passage of amino-acids across the cell-wall into the cell may be by a process of diffusion, as in the case of lysine, or may require energy drawn from exergonic metabolism, as in the case of glutamic acid. Gale and Taylor (1947) have shown that the assimilation of glutamic acid by *Staph. aureus* is blocked if the cells grow in the presence of penicillin. Taylor (1947) was unable to demonstrate the presence of free amino-acids within Gram-negative cells, and there is as yet no knowledge of the nature of amino-acid assimilation in such organisms, or whether the processes involved are affected by penicillin. Pollock and Wainwright (1948) showed that the rate of adaptation to nitrate of a coliform organism was markedly influenced by the nature of the nitrogen source available. The formation of the adaptive nitratase was increased by the addition of amino-acids, a variety of amino-acids being more effective than a single one, and maximal adaptation occurring when a protein hydrolysate was supplied as N-source. It is clear that the rate of nitratase production could be used as an indirect measure of the assimilation of amino-acids. This work was accordingly undertaken in an attempt to study some properties of amino-acid assimilation in this Gram-negative organism and to investigate the action of penicillin on the assimilation. It is shown below that, although an effect of penicillin on adaptation was discovered, it is probable that this action is not related to amino-acid assimilation or protein synthesis. The results are inexplicable in the present state of knowledge, but are recorded since they may be related in general to studies of penicillin action.

ORGANISM AND METHODS.

The organism used was the coliform "1433" used by Pollock and Wainwright (1948). It was maintained on casein-digest-agar slopes. The organisms used in experiments quoted below were grown on the surface of casein-digest agar for 16 hours at 30° C. The organisms were harvested, washed and suspended in a salt solution of the following composition: 3·0 g. KH_2PO_4, 10·0 g. Na_2HPO_4, 3·0 g. NaCl, 2·1 g. $MgSO_4$, $7H_2O$, 1000 ml. distilled water.

Methods used for the estimation of nitrite, nitratase activity and for following adaptation to nitrate were as described by Pollock (1946) and Pollock and Wainwright (1948). In principle, nitratase is estimated by determination of nitrite formed from nitrate in the presence of the organisms, with sodium formate as

H-donator; adaptation to nitrate is followed either by estimation of nitrite formed during incubation of the organism with nitrate, glucose and an external N-source, or by estimation of the nitratase activity of the organisms before and after such adaptation. All incubations were carried out anaërobically at 37° C., and quantities of reagents were the same as those used by Pollock and Wainwright (1948).

The penicillin used was a commercial crystalline preparation assayed at 1640 Oxford Units per mg. The vitamin-free casein hydrolysate used was obtained from Ashe Laboratories.

Preliminary investigations.

The adaptation towards nitrate of cells grown in nitrate-free medium was first followed by estimation of the nitrite formed when the cells were incubated in the presence of nitrate and glucose. The acceleration of adaptation by the addition of ammonia or mixtures of amino-acids was confirmed. The effect of the presence of penicillin was tested in systems to which vitamin-free casein-hydrolysate was added as external N-source. No effect on adaptation was observed with penicillin concentrations up to M/5500 (*c.* 100 units per ml.); when concentrations greater than this were present the rate of adaptation began to fall, in comparison with penicillin-free controls, after 90 to 120 min. Since no effect could be observed under 90 min. it seemed probable that some degree of growth must occur in the cells before penicillin exerts any inhibitory effect. The action then takes place under conditions similar to those reported for the action of penicillin on growth, respiration and glutamic acid assimilation in *Staph. aureus* (Chain and Duthie, 1945; Gale and Taylor, 1947). The effect of treating the cells with penicillin before adaptation was next tested. The cells, directly after harvesting, were suspended in the salt solution (final suspension strength = 0·25 mg. dry weight of cells per ml.) containing M/100 NH_4Cl and M/100 glucose. Penicillin was added to various samples at concentrations ranging from 10 to 1000 units per ml. and the cultures then incubated at 37° C. for 2 hours. The cells were centrifuged down, washed once with salt solution and then resuspended in salt, nitrate, glucose and casein hydrolysate solution for adaptation. The course of nitrite production showed that pretreatment with 10 units penicillin per ml. resulted in a slight but definite acceleration of the rate of adaptation, whereas pretreatment with 100 to 1000 units penicillin per ml. resulted in a marked decrease in the rate of adaptation. Parallel experiments were carried out in which the casein hydrolysate was omitted during adaptation, and it was found that the course of nitrite production was apparently inhibited in the same proportion as in the presence of casein hydrolysate. It was difficult to gauge the relative inhibitory effects in the various media by this method, and further experiments were made by estimating the nitratase in the washed cells before and after adaptation.

General procedure.

The cells were washed from the surface of agar in *c.* 10 ml. salt solution, centrifuged down and resuspended in salt solution to a final strength of 1·0 mg. dry weight of cells per ml. To x ml. of suspension were then added $3x$ ml. salt solution containing M/100 NH_4Cl, M/100 glucose and penicillin of the required

concentration. The suspensions were incubated for 2 hours at 37° C., centrifuged down and washed once in $4x$ ml. salt solution. The washed cells were then suspended in x ml. salt solution and added to $3x$ ml. salt solution containing M/100 $NaNO_3$, M/100 glucose and 0·1 per cent casein hydrolysate or other N-source as described below. A sample of unadapted cells was tested for nitratase activity, and the remainder incubated for 90 min. at 37° C. in an atmosphere of nitrogen. The cells were then centrifuged down, washed once in $4x$ ml. water and their nitratase activity determined. Until the final stage the cells were kept in salt solution and, except when on the centrifuge, at 37° C. All experiments were controlled by estimations on cells treated in the same manner, but with the omission of penicillin during the first period of incubation.

RESULTS.

Inhibition of Nitratase Formation by Penicillin.

Effect of penicillin concentration.

Incubation of non-adapted cells with glucose and nitrate results in an increase of nitratase activity even in the absence of an added source of nitrogen. The effect of pretreatment with penicillin was to reduce the rate of this increase. Fig. 1 shows the degree of inhibition obtained by pretreatment with various

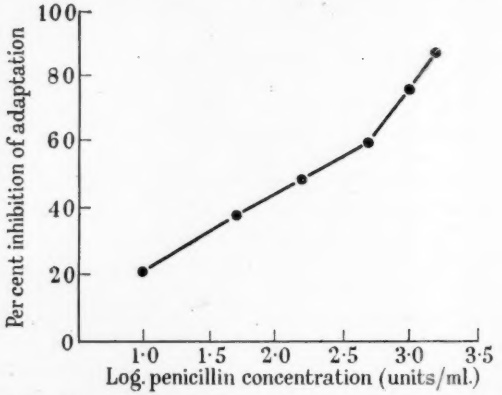

FIG. 1.—Relation between penicillin concentration during pretreatment and subsequent inhibition of nitratase adaptation in organism 1433.

Cells incubated for 2 hr. at 37° C. in salt solution containing M/100 NH_4Cl and M/100 glucose with penicillin concentrations as abscissae. Cells centrifuged down, washed once with salt solution and then incubated for 90 min. in salt solution containing M/100 $NaNO_3$, M/100 glucose. Cells centrifuged down, washed in water and nitratase activity determined. Ordinate = nitratase activity of penicillin-pretreated cells expressed as per cent activity of cells treated by same procedure in absence of penicillin.

concentrations of penicillin ; 50 per cent inhibition is obtained by pretreatment with *c.* 500 units penicillin per ml. The curve relating inhibition to penicillin concentration shows an apparent discontinuity at the 60 per cent inhibition level, and it was observed that cells which had been treated with concentrations of penicillin greater than 600 to 700 units per ml. showed a tendency to aggregate.

It is possible that the greater inhibition obtained with higher penicillin levels was due to a secondary effect of the cell aggregation.

The organism 1433 possesses penicillinase, so that it is not possible to determine the concentration of penicillin inhibiting growth in a nutrient medium. When tested by the usual serial dilution method, using an inoculum of *c.* 10^6 cells in casein-digest medium, growth was checked by 500 units penicillin per ml. for 24 hours but took place at 1000 units per ml. in 48 hours.

Effect of external N-source.

Cells were pretreated with 500 units penicillin per ml. and then allowed to adapt to nitrate in the presence of various external nitrogen sources such as ammonia, lysine, glutamic acid, or an amino-acid mixture represented by vitamin-

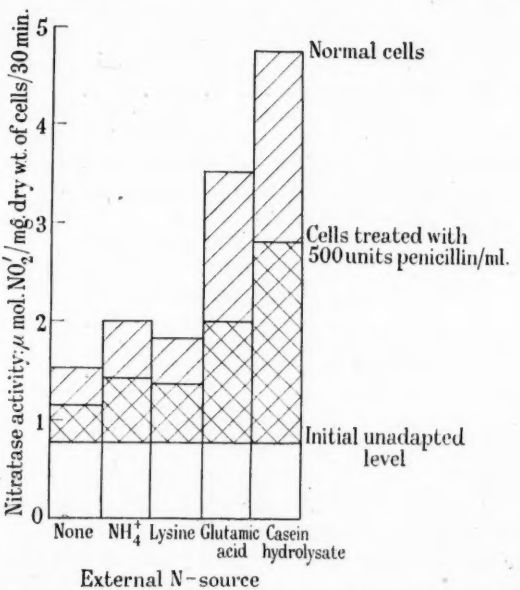

FIG. 2.—Variation of nitratase adaptation of normal and penicillin-treated cells with nature of external N-source.

Cells incubated for 2 hr. at 37° C. in salt solution containing M/100 NH_4Cl and M/100 glucose with 500 units penicillin/ml. (no penicillin in controls = normal cells). After washing free from penicillin, cells incubated for 90 min. at 37° C. in salt solution containing M/100 $NaNO_3$, M/100 glucose and 1 per cent N source as above.

free casein hydrolysate. In each case the adaptive increase was compared with that which had taken place in cells pretreated in the same way but in the absence of any penicillin. Fig. 2 shows that, whatever the available N-source, the pretreatment with 500 units penicillin per ml. has resulted in 50 per cent inhibition of the adaptive increase in nitratase activity. There is no evidence

that the adaptive increase stimulated by, say, lysine is affected differently from that stimulated by glutamic acid. The results suggest that it is the capacity to adapt which is affected by penicillin treatment rather than the assimilation of the available N-source.

TABLE I.—*Absence of Action of Penicillin on Glucose Fermentation.*

Organism 1433 grown for 16 hr. at 30° C. on casein-broth-agar ; harvested and washed once in salt solution. Fermentation studied in Warburg manometers containing 1·0 ml. M/5 phosphate buffer pH 7·0, 1·0 ml. suspension of organism (= 5 mg. dry weight of cells), 0·5 ml. M/100 glucose, 0·5 ml. water or penicillin solution as below ; manometers filled with O_2-free N_2 ; CO_2 absorbed by 0·25 ml. 10 per cent NaOH in centre pot. Fermentation activity expressed as Q_{H_2} = µl. H_2 evolved/hr./mg. dry weight of cells.

Cell preparation.	Penicillin concentration (units/ml.).	Q_{H_2}.
(1) Washed cells after harvesting	0	11
	500	11
	1000	8·5
(2) Cells incubated for 2 hr. at 37° C. in salt solution containing M/100 N_4HCl and 0·01 M glucose, washed and made up into suspension as (1) . . .	—	31
(3) Incubated as in (2) but in presence 500 units penicillin/ml.	—	40
(4) Incubated as in (2) but in presence 1000 units penillin/ml.	—	39
(5) Cells incubated and prepared as in (4) but tested in presence M/20 adenosinetriphosphate . . .	—	37

Effect of penicillin pretreatment on glucose fermentation.

The energy for adaptive formation of nitratase, under the experimental conditions used, is derived from glucose fermentation. It is shown in the table that there is no significant difference between the rates of fermentation of cells whether pretreated with penicillin or not. Consequently the impairment of the adaptive capacity cannot be due to inhibition of the energy-supplying system.

Reversal of the Penicillin Effect.

Action of adenosine-triphosphate.

The penicillin concentrations effective in these experiments are approximately the same as those which Gros and Macheboeuf (1948) find inhibit the breakdown of adenosinetriphosphate (ATP) and other nucleotides by *Cl. sporogenes*. It seemed possible that, if the adaptive capacity were affected by an energy-transferring mechanism, then nucleotide metabolism might be involved, and that modification of the penicillin effect could be obtained by adding ATP or related compounds to the cells during adaptation. Cells were treated with 500 units penicillin per ml. and their rate of adaptive increase determined as before, and also in the presence of concentrations of ATP ranging from 1 to 10 mg. Ba salt

per ml. The higher concentrations of ATP were found to abolish the inhibitory action of penicillin. Fig. 3 shows the effect of ATP concentration on the adaptation of both normal and penicillin-treated cells; in the normal cells low concentrations of ATP increase the rate of adaptation, but high concentrations are slightly inhibitory; in the penicillin-treated cells low concentrations of ATP increase the inhibition, whereas the inhibition is reversed and completely

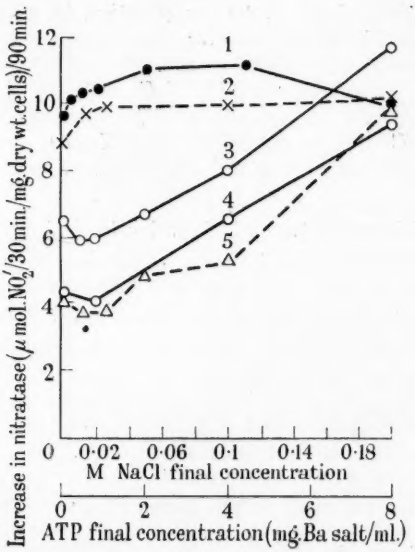

FIG. 3.—Effect of adenosinetriphosphate preparation and of salt in removing penicillin inhibition of nitratase formation.

Cells incubated for 2 hr. at 37° C. in salt solution with NH_4Cl and glucose and penicillin as before (see Fig. 2). After washing in salt solution, cells incubated for 90 min. at 37° in salt solution containing M/100 $NaNO_3$, M/100 glucose, 1 per cent casein hydrolysate, and either ATP solution or NaCl to the final concentrations indicated. Cells then washed in water and increase in nitratase activity determined.

Curve 1. Normal cells in presence of ATP preparation.
" 2. Normal cells in presence of NaCl.
" 3. Cells pretreated with 500 units penicillin per ml. and adapted in presence of ATP preparation.
" 4. Cells pretreated with 700 units penicillin per ml. and adapted in presence of ATP preparation.
" 5. Cells pretreated with 700 units penicillin per ml. and adapted in presence of NaCl.

abolished by higher concentrations. The amount of ATP necessary to abolish the inhibition increases with the degree of inhibition, i.e. with the concentration of penicillin used for pretreatment. It was immediately obvious that the concentrations of ATP which are fully effective in abolishing the penicillin effect are very high, representing a weight of ATP many times greater than the weight of organism present. It seemed improbable that the effective agent in the ATP preparation was ATP itself. Preparations of adenylic acid, adenine, adenosine, ribose, etc. were completely inactive. When the ATP preparation was

subjected to 10 min. hydrolysis in N/1 HCl, and neutralized before test, it was found that its effectiveness in abolishing the penicillin effect was increased. This suggested that the anti-penicillin action resided in the salt content of the preparation.

Action of salts.

Fig. 3 shows also the effect of adding NaCl to the reaction mixture during adaptation. The action of the ATP preparations in abolishing the penicillin effect can be reproduced in all respects by suitable concentrations of NaCl. The ATP solution used contained a high concentration of salt, and in Fig. 3 the ATP ordinates are made to correspond with the NaCl ordinates for like salt concentration; the action of the ATP preparation is fully explained by its salt content.

The action of NaCl can be reproduced by Na_2SO_4 or KCl, similar actions being produced by solutions of similar ionic strength.

Conditions for Reversal of Penicillin Action by Salt.

The experiments described so far were all carried out with cells which were first treated with penicillin and then allowed to adapt in the absence of penicillin. Salt was added during the adaptation process. Fig. 4 shows that—

(1) The presence of salt during the treatment with penicillin does not prevent subsequent inhibition of adaptation.

(2) After pretreatment with penicillin, salt abolishes the inhibition if present during the adaptation in the absence of penicillin.

(3) If the cells are first treated with penicillin, then with salt, then washed and allowed to adapt in the absence of either salt or penicillin, the inhibitory action of penicillin is largely abolished.

(4) If the cells are treated with penicillin, then allowed to adapt in the absence of penicillin or salt, and the adapted cells then treated with salt, the salt has no action in removing the inhibitory effect due to penicillin after adaptation has taken place.

Consequently the antagonistic action of salt is shown only when the salt is added after the removal of the penicillin, and prior to or during the adaptation process.

It was noted above that treatment of the cells with high concentrations of penicillin often resulted in aggregation of the cells during later stages of the experiment. This was particularly noticeable when penicillin-treated cells were incubated in the presence of casein hydrolysate (for the adaptation process). It was always found that the tendency to aggregate was lost whenever treatment with salt abolished the penicillin effect on adaptation.

Experiments with Staphylococcus aureus.

Assimilation of glutamic acid.

Gale and Taylor (1947) found that if *Staph. aureus* were grown in a medium to which penicillin was added 90 min. before harvesting, the cells lost the ability to assimilate glutamic acid. It seemed possible that this effect might also be affected by salt. Consequently, *Staph. aureus* was grown in the presence of

1 unit penicillin per ml. under conditions identical with those previously described by Gale and Taylor (1947), and the ability of the cells to assimilate glutamic acid was tested with and without treatment with M/5 NaCl. Penicillin treatment completely blocked the passage of glutamic acid into the cells and this effect was not reversed by treatment with salt.

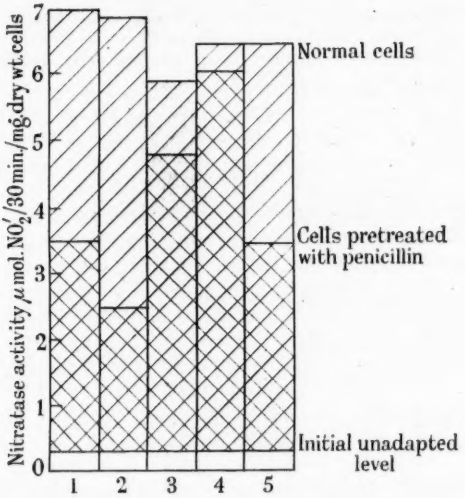

FIG. 4.—Effect of salt on penicillin inhibition of nitratase formation.

Cells pretreated with penicillin and then allowed to adapt in the presence of nitrate, glucose and casein hydrolysate.

1. Cells pretreated with 700 units penicillin per ml. Adaptation process as usual.
2. Cells pretreated with 700 units penicillin per ml. in presence of M/5 NaCl; adaptation process as usual.
3. Cells pretreated with penicillin; washed; incubated for 30 min. in M/5 NaCl; washed; adaptation process as usual.
4. Cells pretreated with penicillin; washed; adaptation process in presence of M/5 NaCl.
5. Cells pretreated with penicillin; washed; adaptation process as usual; washed; incubated 30 min. with M/5 NaCl before testing.

Controls (= normal cells) treated in parallel fashion in all cases in absence of penicillin during first stage.

Nitratase adaptation.

Staph. aureus cells possess nitratase, and will undergo adaptation in a manner similar to that described for the organism "1433." The adaptation is stimulated by the addition of single amino-acids as N-source. Cells were grown in penicillin (1·0 unit per ml.) until their ability to assimilate glutamic acid was lost as above. It was found that glutamic acid would still stimulate adaptive nitratase formation.

DISCUSSION.

No explanation of the effects described in this paper can be put forward at the present time. During the course of the work it seemed clear that there

was some connection between the tendency of the cell suspensions to aggregate and an inhibition of the rate of adaptation. However, inhibitory effects were noted after treatment with penicillin in concentrations (100 units per ml. and less) which did not give rise to later aggregation of the cells. The suspensions were examined under phase-contrast lenses, and, except where obvious aggregates were found after treatment with high concentrations of penicillin, the cells were motile and of the same appearance under all the conditions described in this paper. It seems probable that the treatment with penicillin produces some alteration in the surface of the cell, which is reversed by salt solution, and which is reflected in a slowing of the rate of adaptation to nitrate. Such effects might be expected if nitratase were an enzyme residing in or near the surface of the bacterial cell. There is no definite evidence that this is the case, although if the cells are disintegrated by mechanical agitation, nitratase activity is always associated with the solid particles (Pollock, private communication). The fact that penicillin does not differentiate between ammonia and the various amino-acids as N-sources for adaptive protein formation might also be explicable if the enzyme were a surface one. The experiments carried out with *Staph. aureus*, in which it was found that glutamic acid stimulates nitratase formation although its assimilation has been blocked by penicillin, would also be explicable if the site of nitratase synthesis were situated in the cell-surface at a level outside that of the osmotic barrier which normally restricts the entry of glutamic acid into the cell.

It is clear that these effects deal with problems of enzyme location and of the nature of the bacterial surface, and explanations must await further developments in these fields.

SUMMARY.

(1) Incubation of cells of the coliform organism " 1433 " with high concentrations of penicillin results, after removal of the penicillin, in a decrease in their rate of adaptation to nitrate.

(2) This effect of penicillin can be removed if the cells are incubated in the presence of high concentrations of salt prior to, or during, the adaptation process. The presence of salt during the initial treatment with penicillin does not remove the effect. Treatment of the cells after adaptation has occurred does not restore the full nitratase activity.

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